THE EXPRESSION OF MURINE LEUKEMIA VIRUS PROTEINS

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PROEFSCHRIFT

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Dit proefschrift kon slechts tot stand komen dankzij de hulp van velen.
Het persoonlijk voornaamwoord "we" in de beschrijving der experimenten staat er niet als "pluralis modestiae", maar geeft de essentiële bijdrage weer van mw. Philomeen Hagenberg, mw. Jet van Eenbergen en mw. Annemiete van der Kemp, die op zo goede en toegewijde wijze de meeste van de beschreven experimenten hebben uitgevoerd. De positief-kritische instelling van Annemiete heeft veel bijgedragen tot de vormgeving van het onderzoek.
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Aan allen die mij hielpen

CONTENTS

NOMENCLATURE OF VIRAL POLYPEPTIDES

To indicate viral proteins and (precursor) polyproteins a generally accepted nomenclature is used (1,2).

Each polypeptide is designated by its apparent molecular weight in thousands, taken from gel filtration in 6 M guanidine hydrochloride for polypeptides below MW 30,000 and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) for polypeptides above MW 30,000. (Breaking this rule, sometimes SDS-PAGE results are also used to indicate apparent molecular weights of the smaller viral proteins).

The number indicating the apparent molecular weight of the polypeptide is preceded by a "p" for protein, "gp" for glycoprotein, and "pp" for phosphoprotein or by "P" for polyprotein, "gP" for glycosylated polyprotein, "Pr" for precursor polyprotein, "gPr" for glycosylated precursor polyprotein and "pPr" for phosphorylated precursor polyprotein.

Additional information about the structural role of a viral protein can be added in parentheses, e.g. (E) indicates an envelope protein, (C) a core protein and (N) a ribonucleoprotein. (Note: parentheses are often omitted). In practice the use of these additions is restricted to the murine leukemia virus envelope proteins p15E and p12E, to discriminate them from the unrelated core proteins p15 and p12.

(Precursor) polyproteins are identified further by a superscript indicating the gene (gag, pol, env or onc/src) from which they originate.

A proposed nomenclature (3) that uses the subgene order of each viral protein for its designation (e.g. p15 = gag-a, p12 = gag-b, etc.) is not (yet) adopted by most authors.

Examples:

p15 : viral core protein of 15,000 apparent molecular weight

p15(E) or p15E	: viral envelope protein of 15,000 apparent molecular weight
gp70	: viral envelope glycoprotein of 70,000 apparent molecular weight
$gP80^{gag}$	: glycosylated gag polyprotein of 80,000 apparent molecular weight (containing viral core proteins p15, p12, p30 and p10)
$gPr82^{env}$	: glycosylated env precursor polyprotein of 82,000 apparent molecular weight (containing viral envelope proteins gp70 and p15E)
$pPr65^{gag}$ or $Pr65^{gag}$	: phosphorylated gag precursor polyprotein of 65,000 apparent molecular weight

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Note: In chapters 5 and 7 we introduce a similar but not identical nomenclature for still unidentified virus-related translation products: a number indicating apparent molecular weight in thousands preceded by "tp" for translation product.

1. INTRODUCTION AND PERSPECTIVE

INTRODUCTION AND PERSPECTIVE

The murine leukemia viruses belong to the RNA tumor virus group (generic name: Oncovirinae; see chapter 2.1.). These viruses are widespread among vertebrates. In 1970 Temin et al. (1) and Baltimore (2) simultaneously and independently discovered a unique feature of these viruses: they contain an enzyme, called reverse transcriptase or RNA-dependent DNA polymerase, that is able to transcribe RNA into DNA. This discovery not only led to some theoretical discussions about Crick's "central dogma" (3). More importantly, a main tool came available for the construction and analysis of genetic recombinants that contain eukaryotic genes integrated in prokaryotic vehicles (e.g. 4,5). The discovery of reverse transcriptase also greatly stimulated further research of the architecture, the replication and the gene expression of RNA tumor viruses, and, of course, of the mechanism by which they induce tumors. Particularly their role in the etiology of cancer in humans has been the subject of intensive research (6). Temin and Baltimore received the Nobel price in 1975.

In the past decade most details about the composition and structure of the RNA tumor viruses and about their replication and gene expression have been elucidated. These subjects are discussed in the sections 2, 3 and 4 of chapter 2. The work on the gene expression of RNA tumor viruses was placed in an ordered frame by Baltimore (7) who in 1973 defined four genes on the non-defective RNA tumor virus genome: a gene encoding the viral core proteins, a gene encoding the viral envelope proteins, a gene coding for reverse transcriptase, and a gene responsible for the transforming and tumorigenic properties of the virus. The prototype virus for this gene classification was the Rous avian sarcoma virus (ASV). This virus rapidly induces sarcomas in chickens and transforms fibroblasts in vitro. On the other hand, avian leukosis viruses and murine leukemia viruses only induce tumors in haemopoietic

tissues after a delayed period of time and are not able to transform fibroblasts. The idea grew that these viruses lack a separate gene for oncogenesis.

Earlier work in this field from this laboratory primarily dealt with the biosynthetic pathway of viral structural proteins in murine leukemia virus-infected cells on the one hand and with the virus-specific RNAs in these cells on the other (8,9,10). The translation products of virion RNA have also been studied (11). It was found that infected cells contain different size-classes of virus-specific mRNAs. In this thesis (chapters 3, 4, 5) experiments are described that show that each of these size-classes directs the synthesis of different virion constituents. Core proteins and reverse transcriptase are synthesized on genome-length mRNA, whereas the envelope proteins are made on a subgenome-size mRNA, comprising about half of the genome. A still smaller mRNA directed the synthesis of cellular proteins that in some way are encapsidated by the virions.

These results were obtained by translating size-fractionated mRNA from murine leukemia virus-infected cells in oocytes of the African clawed frog Xenopus laevis. These oocytes are large enough for injection by simple technical means and have a major advantage compared to cell-free translation systems in that all cellular processes are continuing apparently unimpaired. The oocyte system has been reviewed recently by an other thesis from this laboratory (12).

Since it was known from the literature that the subgenomic mRNA directing the synthesis of murine leukemia virus envelope proteins arises from genome-length RNA in the nucleus of the infected cell (see chapter 2.4.4,5), we reasoned that injection of genome-length RNA from the virus itself, from the cytoplasm or from the nucleus of the infected cell into the nuclei of Xenopus oocytes possibly might also lead to the formation of a subgenomic mRNA that supports the synthesis of viral envelope proteins. These experiments are

described in chapter 7. Similar experiments, described in the literature, using an other, highly sensitive system, did have success, but we were unable to reach the same result with the oocyte system. Possible reasons are discussed.

About the mechanism by which RNA tumor viruses induce tumors much less is known. Only the sarcoma-induction by avian sarcoma viruses is rather well-documented. Despite all efforts, involvement of RNA tumor viruses in the etiology of human cancer could not be demonstrated convincingly (6).
As to leukemia induction by leukemia viruses, the importance of recombination by these viruses with endogenous viral sequences was recognized. These recombinations seem to occur rather frequently and not only with more or less related viral sequences. Probably they occur at random with at least part of the host genome. The vast majority of these recombinations presumably are dead ends, but in rare cases some viral sequences are replaced by host sequences, either virus-related or not, which transfer a new biological property to the newly formed variant. With the aid of appropriate in vitro selection techniques (13) these variants may prove to be a general tool for cloning of cellular genes (14). Variants that acquire (extra) tumorigenic properties will be positively selected by the organism. As a consequence of the replacement of viral by non-viral sequences the virus becomes replication-defective. Examples of this kind of RNA tumor viruses are the avian acute leukemia viruses and the mammalian sarcoma viruses. ASV, the prototype RNA tumor virus, as a non-defective sarcomagenic virus in fact appears to be an exceptional member of the RNA tumor virus group!
Recombinant viruses are discussed in chapter 2.4.5.

A still rather undeveloped field in RNA tumor virology is the immunological defense of the organism against the virus and the virus-induced tumors. In this respect a major role may be played

by the virus-related cell surface antigens that are present on infected, and in some cases, uninfected cells.
Chapter 2.7. deals with the present knowledge of these antigens. One example of the virus-related cell surface antigens is the Moloney cell surface antigen (MCSA) that is related to a tumor-specific transplantation antigen induced on mouse cells by Moloney murine leukemia virus. The nature of this antigen was the subject of a number of studies but has been unknown for a long time. In chapter 6 of this thesis it is shown that MCSA is a product of the envelope gene of a defective virus that is not related to the Moloney strain of murine leukemia viruses.

It is not difficult to foresee that in the near future many puzzling aspects in RNA as well as DNA tumor virology will be solved. The combination of the recombinant DNA technique and sophisticated sequence techniques will reveal many data on gene structure and expression both of viruses and of cells. Molecular biology and immunology alike will profit from the newly developed hybridoma technique for the generation of monoclonal antibodies. For this reason, the prospects for a better understanding of oncogenesis and the role of immunological mechanisms in the defense against cancer seem very favourable at the moment.

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2. RNA TUMOR VIRUSES – A SURVEY

RNA TUMOR VIRUSES – A SURVEY

In this outline emphasis will be laid on the description of murine leukemia viruses. However, attention will be paid to other types of murine RNA tumor viruses and to RNA tumor viruses of other species, especially of birds, whenever applicable.

This survey is meant to indicate the scientific frame in which the experimental work reported in this thesis fits. Therefore, rather than giving another review of the (murine) RNA tumor viruses, I decided to refer quite often to some recent reviews that give a complete account of the vast amount of original papers. This enabled me to concentrate on the newest findings not yet covered by published reviews in this rapidly expanding field.

2.1. MAIN CHARACTERISTICS AND CLASSIFICATION

Taxonomically RNA tumor viruses (Oncovirinae) form the main subfamily of the virus family called Retroviridae (82).
Retrovirus particles have a diameter of about 100 nm. They consist of a lipoprotein envelope with glycoprotein protrusions ("spikes") enclosing an icosahedral protein core shell within which there is a helical nucleocapsid. Recently a thin layer, the inner coat, has been detected just beneath the viral envelope (30,196). The most characteristic biochemical feature of the family is the occurence of the enzyme RNA-dependent DNA polymerase (reverse transcriptase) within the virion (82,312). The RNA content of a virus particle is about 1 %, protein 60-70 %, lipid 20-30 % and carbohydrate 2 % (312). The viral genome is a single stranded RNA molecule with a molecular weight of about 3×10^6 which is present in the nucleocapsid as a dimer (240) complexed with tRNA (307; cf. section 2.2.5.).

A confusing array of taxonomic devices has been developed to classify the RNA tumor viruses.
1. Morphology. On the basis of their morphology RNA tumor viruses can be classified as type A, type B, type C or type D viruses (55, 312).
Type A viruses are virus-like particles with a toroidal nucleoid. They are believed to be precursors of B-type and C-type viruses. Cells releasing B-type particles contain A-type particles, enclosed by a core membrane, in their cytoplasm. The A-type particles move from the endoplasmatic reticulum to the cell surface, from which they bud, acquiring an outer membrane with regularly arranged spikes. The toroidal nucleoid is then converted into the spherical and eccentrically located nucleoid of the mature B-type particle. The C-type particles develop quite differently. Their nucleocapsids first appear as crescent-shaped

structures immediately below the cell surface and as the virion buds from the cell surface the nucleoid matures into a complete toroid surrounded by an inner core shell and an outer envelope membrane. Because of the toroidal nucleoid these particles are also classified as A-type particles. They further develop into the mature C-type particles with their spherical, centrally placed nucleoid.

Finally, D-type viruses have a larger diameter than B- or C-type particles and have pleomorphic nucleoids.

The most prominent representative of the B-type viruses is the mouse mammary tumor virus (MuMTV). The Mason-Pfizer monkey virus (MPMV; an endogenous virus of the langur monkey) is one of the few known D-type viruses. C-type viruses have been isolated from reptiles, birds and mammals. Especially from chickens and mice a number of C-type virus isolates have been well characterized.

2. Transmission (312). Exogenous RNA tumor viruses persist by horizontal or epigenetic spread (e.g. through the milk) among members of a susceptible species. Endogenous viruses spread by vertical transmission of the viral genome, integrated as provirus in the host genome, through the gametes from parent to offspring.

3. Species of origin. RNA tumor viruses are commonly identified according to the species from which they have been first isolated. Occasionally virus strains were isolated from what appeared to be a secondary host (21,22,182,312). For murine endogenous viruses even the particular mouse strain they are isolated from is used for their classification.

4. Pathogenicity (108,312). RNA tumor viruses can be named or grouped after the kind of neoplasia they induce. Carcinoma viruses cause malignant tumors of epithelial origin. Sarcoma viruses cause solid connective tissue tumors. A number of different tumors of the haemopoietic and reticulo-endothelial systems can be induced by RNA tumor viruses. These tumors can either be leukemic, with highly elevated levels of white blood cells (e.g. lymphatic leukemia, erythroblastosis) or aleukemic, with a solid tumor in

various compartments of the reticulo-endothelial system (e.g. lymphosarcoma, thymoma).

Generally a clonal isolate causes only one kind of tumor. Some transforming RNA tumor viruses, however, like the murine sarcoma viruses, are replication-deficient and need non-defective leukemia viruses for their replication (326). So-called pseudotypes are formed: the helper virus supplies the necessary structural and non-structural proteins not coded for by the sarcoma viral genome (326). Finally, absence of any known pathogenicity is also found for some RNA "tumor" viruses that in all other respects are closely related to pathogenic RNA tumor viruses.

5. Host range (312). Differences in host range form the basis of the sub-division of avian viruses in seven main subgroups (A to G). Murine leukemia viruses are grouped in ecotropic (only infectious for mouse cells), xenotropic (not infectious for mouse cells, but infectious for cells of several other species) and amphotropic viruses (infectious both for mouse cells and for cells of other species).

Eco- and amphotropic viruses can be N-tropic (1000 or more times preferential growth in cells carrying the so-called $Fv\text{-}1^{n/n}$ haplotype, typically represented by the NIH-Swiss cell line), B-tropic (preferential growth in cells with a $Fv\text{-}1^{b/b}$ haplotype; e.g. BALB/c cells) or NB-tropic (growth in both types of cells). The Fv-1 gene and other host genes (among them genes coding for receptors on the cell membrane that recognize viral envelope glycoproteins) determining the host range of RNA tumor viruses, are the subject of extensive research (26,97,122,291).

6. Antigenicity (6,298,312). Three major forms of antigenicity are associated with RNA tumor viruses. Type-specific antigens identify each particular strain of virus. Group-specific antigens are shared by related viruses from a single species and inter-species antigens are the (few) antigens that viruses from different species have in common. These three classes of antigenic specificity are present in different proportions on all viral

proteins. By competitive radioimmunoassays (RIA) one can group the viruses according to the antigenic relatedness of each of their structural (39, 123, 124, 182, 294, 296, 303, 304) or non-structural (106, 219) proteins.

Rather soon it was recognized (312) that the main type-specific antigen of avian viruses, present on the viral envelope glycoprotein, could be used as a serologically detectable marker for the avian subgroup classification. Together with other evidence (312) this has led to the conclusions that the host range of avian tumor viruses was determined by the envelope glycoprotein.

The very recent development of monoclonal antibodies against viral proteins (184, 302) provides a new tool for a more detailed serological classification of the RNA tumor viruses.

7. Biochemical approaches. The isolation of all viral constituents of the virus particle to apparent purity and the rapid progress in technology (itself having been stimulated by the intensive study of RNA tumor viruses all over the world) enabled the use of biochemical characterization as an aid for grouping of viruses. Tryptic peptide mapping of viral proteins (68), RNAse-fingerprinting of viral RNA (340), molecular hybridization with viral RNA or proviral DNA (20, 21, 22, 23, 34, 182), analysis of *in vitro* reverse transcription products (114), all these biochemical methods have been used for classification of RNA tumor viruses. Biochemical analyses have also led to the recognition of molecular differences that coincide with macroscopic differences. For instance, by protein analysis (SDS-polyacrylamide gel electrophoresis, peptide mapping; 96, 126) as well as by RNA analysis (fingerprinting, partial sequence elucidation; 73, 247, 276) it was found that the recognition site for the restrictive action of the Fv-1 locus determining N/B tropism of ecotropic mouse viruses was situated on the core protein p30, while the broader host range (eco-, xeno-, amphotropism) of mouse viruses appeared to be determined by the envelope glycoprotein gp70 (12, 69, 313).

2.2. ARCHITECTURE AND COMPOSITION OF THE VIRION

Most proteins found in the virion are virus-specific and coded for by the viral genome. To the structural components of the virus also belong carbohydrates and lipids. These components are derived from the infected cell. In addition to the viral reverse transcriptase a number of cellular enzymatic activities have been found associated with the virion (312). Several species of RNA can be isolated from the virion. Apart from the high molecular weight RNA (35S), these are cellular RNAs with no apparent role in virus replication, with the exception of a particular tRNA species which plays an important role in reverse transcription of the viral genome (cf. section 2.3.).
A schematic structure of the virion is drawn in Fig. 1. An enumeration of the properties of structural and non-structural virus-specific proteins is presented in table 1. A detailed discussion, also covering the viral genome, is given below.

2.2.1. VIRAL ENVELOPE

The envelope of retroviruses is derived from the plasma membrane of the host cell. Therefore, the viral envelope proteins at the budding sites of the host cell should be arranged in a similar configuration as in the virion. As a result, one may expect to find some cellular plasma membrane constituents in the viral envelope. For instance, a specific histocompatibility antigen and the viral envelope glycoprotein of Friend erythroleukemia virus were reported to be topographically linked on the surface of cells transformed by this virus and virions produced by these cells contained the same antigen (36). The viral envelope, however, mainly consists of three virus-coded proteins. In the murine leukemia virus these proteins are a glycoprotein of 70,000 molecular weight,

gp70, a non-glycosylated protein, p15E, with an apparent molecular weight of 15,000-18,000,and a specific degradation product of p15E with a molecular weight of 12,000-14,000, p12E (67,142,196,298). It should be noted here that similar proteins of different viruses may differ in their molecular weights to a small extent.
The glycoprotein gp70 is the most exposed protein of the virus. It forms the major target for virus neutralizing antibodies (134,141, 290). It contains the main type-specific antigenic determinant (6), it is responsible for host range restriction of the virus (cf. section 2.1.) and it is required for entry of the virus into a host cell (25,58,194,235).
The carbohydrate moiety of the molecule has no or little antigenic activity (29). The protein backbone of the glycoprotein has a molecular weight of about 45,000 (187,349).
The glycoproteins form the "knobs" of the spikes on the virion. The "stalks" are presumably formed by p15E and p12E (30,195,196). The gp70 and p15E/p12E molecules are rather stably associated and partly coupled by covalent disulfide linkages (172,229,230,305,346; H.P.J. Bloemers, personal communication).
One spike is composed of four to six gp70-p15E/p12E subunits (231, 305). The relevance of the proteolytic degradation of p15E into p12E is not clear. This degradation takes place after the release of the virion from the cell (H.P.J. Bloemers, personal communication).

2.2.2. INNER COAT

The composition of the inner coat remains to be established. Bolognesi and coworkers suggest that the viral low molecular weight phosphoprotein is a likely candidate for the formation of this substructure (30,195,196). This protein, pp12, has a molecular weight of 12,000 in rodent viruses, but the protein with similar antigenic and biochemical properties has a molecular weight of

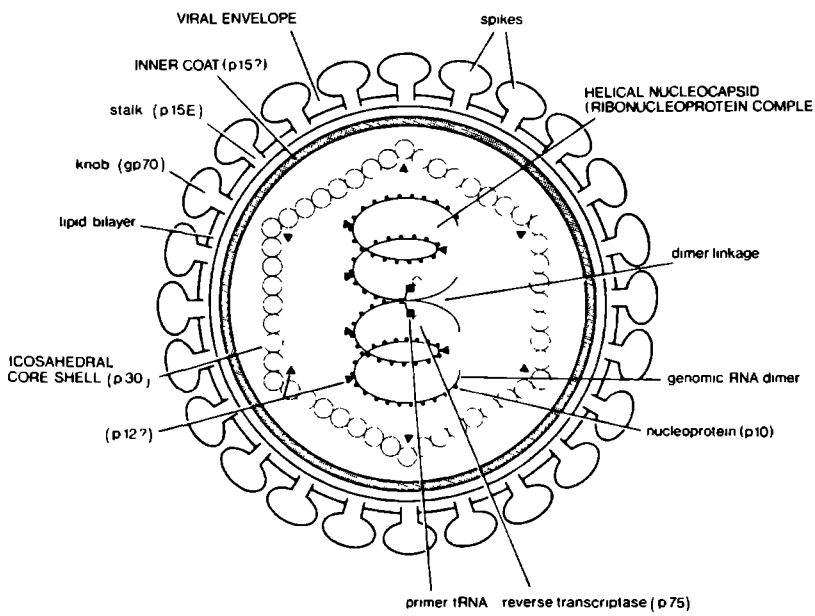

Figure 1. Murine leukemia virus.
Diagrammatic illustration of virion structure and composition.

15,000 (pp15) in some viruses of primate origin. The idea of Bolognesi et al. for the location of pp12 in the inner coat is mainly based on their observation that pp12 could neither be found in purified cores of Friend murine leukemia virus (F-MuLV) nor on the virus surface (30,196). These results are opposed to those of others (216), who detected all four non-envelope structural proteins, including pp15, of the primate virus RD-114 in the viral core particle. Moreover, viral core particles and virions contained these proteins in the same relative amounts. This discrepancy could be explained by a difference in the viral core isolation procedure, giving rise to the cosegregation of the inner coat with the viral core in the latter case. There are indications that the hydrophobic viral protein of 15,000 molecular weight, p15, is rather closely associated with the viral envelope. First, isolated viral envelopes contained 40 % of the amount of p15 present in the intact virion (322). Second, the p15 molecule exhibits typical properties of a membrane protein (13). And, finally, it could be labeled by lactoperoxidase iodination of the intact virion although virus could not be neutralized nor lysed by anti-p15 antibodies (13). These results suggest a localisation of p15 just below the virus surface and perhaps p15 is the protein that forms the inner coat.

2.2.3. CORE SHELL

The primary constituent of the core shell is the protein p30 (30,000 molecular weight), the major protein species of the virion. It has strong group-specific and interspecies antigenic determinants (6,196). As discussed before (cf. section 2.1.) this protein determines N/B tropism of murine eco- and amphotropic viruses. The actual localization of pp12 is not known, but most evidence points to a core-associated place for this phosphoprotein. A remarkable feature of this protein is its ability to bind specifically to the homologous genomic RNA, with less than 15 mole-

Table 1.

Structural and non-structural protein components of murine leukemia virus.

Viral protein	% (a)	Structural component	Virion structure	Molecular weight	Isoelectric point	Antigenic determinants (b)	Other properties
gp70	10	"knob" of spike	envelope	70,000	4.5 - 6.2	t,g,(i.s.)	Glycosylated, determines virus penetration and is target for humoral response against virus infection
p15E(c)	2	"stalk" of spike		15,000-18,000	4.4 - 4.6	g,i.s.	Non-glycosylated, hydrophobic
p12E(c)	13			12,000-14,000			
p15	13	inner coat?	inner coat	15,000	6.1 - 8.3	t,g,i.s.	Hydrophobic
pp12	7	core associated?	core exterior	12,000	4.1 - 5.9	t,(g)	Hydrophilic, phosphorylated; binds to homologous RNA
p30	50	core shell		30,000	6.2 - 8.0	(t),g,i.s.	Major viral core protein
p10	4	nucleoprotein	ribonucleoprotein-complex	7,000-10,000	9.1 -11,6	g,(i.s.)	Highly basic, binds to RNA and DNA
p75	1	reverse transcriptase		70,000-80,000	n.t.(d)	g,i.s.	RNA-dependent DNA polymerase

a) Estimate of weight percentage of total virion protein represented by each protein.
b) t = type-specific, g = group-specific, and i.s. = inter species. Minor determinants are in parentheses.
c) In the literature discrimination between p15E and p12E is not always made.
d) n.t. = not tested.

Data are compiled from the literature cited in the text.

cules per molecule of RNA (263,264). This binding depends on the extent of phosphorylation of pp12: only molecules with a low level of phosphorylation bind strongly to the viral RNA (265).

2.2.4. RIBONUCLEOPROTEIN COMPLEX

The viral genome is embedded in a nucleocapsid structure that contains the basic protein p10 (7,000-10,000 molecular weight) as its structural component. This protein can bind RNA and DNA with a marked preference for single-stranded nucleic acid molecules (57, 258). Maximally one p10 molecule per 70 nucleotides can be bound to 35S viral RNA (258).

The viral reverse transcriptase is also present in the nucleocapsid, in an amount of about 20-70 molecules per virion (196). The enzyme is able to complex with p30 molecules (9,205) as well as with tRNA (307). A marked difference exists in the binding of avian and mammalian reverse transcriptase to tRNA. Avian reverse transcriptase binds specifically to the primer-tRNA (218,307; cf. next section), murine reverse transcriptase, however, can bind to all tRNA species of different origin (221,307). A number of reviews have been published (106,257,308,333) on this 70,000-80,000 molecular weight enzyme that reversely transcribes the viral genomic RNA into proviral DNA.

2.2.5. VIRAL RNA

The viral genome is present in the virion as an RNA complex with a sedimentation coefficient of about 50-70S. On heat dissociation several loosely associated 4S and 5S RNAs, two RNA molecules of 30-40S ($3.0-3.5 \times 10^6$ molecular weight) and two strongly bound tRNA molecules are released (240,307,312). The two 35S RNA molecules are identical (15,307) and linked to each other in the 50-

70S complex at their 5' termini by hydrogen bonding between anti-parallel palindromic sequences (18,19,112,280). The 3' end of the 35S genome is polyadenylated (about 200 residues; 18,159). The 5' end possesses the typical cap structure m7G5'ppp5'NmpNp (31,93,248) and methylated adenosines are found at specific internal sites of the RNA (31,61,93). These features are typical for eukaryotic messenger RNAs and, in fact, the viral genome can serve as a messenger for the synthesis of virus-specific polypeptides (cf. sections 2.3. and 2.4.). The viral genome is terminally redundant (45,47,112,261, 301,340). The length of this redundancy is about 16 nucleotides for avian sarcoma virus (ASV) (26) and 60 nucleotides for murine leukemia virus (MuLV) (45). The redundancy is not lost in genetic crosses with strains of ASV possessing different terminal redundancies (340) and it may play an important role during the reverse transcription process (cf. section 2.3.). To each 35S RNA molecule one primer-tRNA molecule of cellular origin is bound (307). In ASV this was shown to be $tRNA^{Trp}$, while in rodent viruses it is $tRNA^{Pro}$ (307). Other tRNA species have been implicated for primate viruses (307). Primer-tRNA molecules are rather stably bound by base pairing with their 3' ends (residues 2-17 for ASV (53) and residues 2-20 for MuLV (226)) to the genomic RNA.

This complex is more stable than the dimer linkage between the 35S RNA molecules in the 70S complex (307). The bound tRNA molecule serves as a primer for DNA synthesis during reverse transcription (307; cf. section 2.3.). When this reaction is carried out in an in vitro endogenous system (i.e. a system with whole virions) so-called strong-stop DNA is preferentially made under certain limiting conditions (81). This is a single-stranded DNA molecule with a discrete, short length. With ASV strong-stop DNA has a length of 101 nucleotides, with MuLV the length is about 135 bases (307). The strong-stop DNA of ASV is sequenced (112,280). Hybridization experiments and direct comparison of the strong-stop DNA sequence with the sequence of 5' terminal RNA oligonucleotides (45,112,280, 307) has led to the conclusion that the strong-stop DNA is a copy

of the 5' end of the genome (not including the ultimate m7G5'p). This result locates the primer binding-site of the ASV genome at residue 104-119 (numbered from the 5' terminus of the viral RNA). In analogy, the primer binding-site of the MuLV genome would be at residues 138-156. With the sequence of the strong-stop DNA and the sequence of the 3' end of $tRNA^{Trp}$ now the first 119 ribonucleotides of the ASV genomic RNA are known (112,280). This sequence shows some remarkable features. There is a region complementary to the 3' end of eukaryotic 18S ribosomal RNA at positions 63-69 of the viral RNA and an AUG codon at positions 83-85, suggesting the presence of a ribosome binding-site (279) and an initiation site for translation in this part of the genome. Furthermore, the presence of a primer-tRNA binding-site directly downstream of this region (see above) led to speculations on a regulatory role for the primer-tRNA on the dual function of the genomic RNA: when tRNA is bound, the RNA would be available for reverse transcription and not for translation and vice versa (cf. sections 2.3. and 2.4.).
In vitro ribosome binding experiments, however, showed that the main ribosome binding-site of the genome was situated between residues 9 and 53 (56).
$Pr76^{gag}$ is the product of the 5' terminal gene of the genome (67; cf. section 2.4.). It was shown by formylmethionyl-$tRNA_f^{Met}$ labeling that $Pr76^{gag}$ is a primary translation product (56,217). The sequence of the first N-terminal amino acids of this polyprotein has been elucidated recently (56,217) and, rather surprisingly, one had to conclude that this sequence is not encoded by the first 119 nucleotides of the ASV genome. Apparently, the synthesis of the product of the 5' terminal gene of ASV is initiated more downstream on the genome. The recent availability of proviral DNA in large amounts obtained by molecular cloning techniques, will enable the rapid elucidation of the nucleotide sequence of the whole genome of several viruses, so that many questions of the type discussed in this section are likely to be solved in the near future.

2.3. VIRAL REPLICATION

The glycoproteins of the viral envelope facilitate the adsorption and uptake of the virus by the host cell. Cells have highly specific receptors for the viral glycoprotein (25,58,194,235). Once the virion is taken up by the cell and the viral RNA is released, the reverse transcription can start. Theoretically, the viral RNA is also free for translation. In fact, after infection with a high multiplicity (MOI) viral RNA was found in an EDTA-sensitive association with polyribosomes (256,282) and one publication reports viral protein synthesis prior to the onset (or in the absence) of proviral DNA synthesis (94). However, in these experiments one could not discriminate between RNA originated from infectious or from non-infectious virus particles. The biological significance, if any, of viral peptide synthesis before proviral DNA synthesis remains obscure.

Presumably, for the transcription of the viral RNA into DNA some unknown cellular functions are required (329), but the virus-coded reverse transcriptase is the main enzyme involved (100,332). Study of the proviral DNA synthesis *in vivo* is hampered by the fact that it constitutes only 10^{-5} of the total DNA in the cell (342). Therefore, part of the assumed mechanism is based on results obtained by *in vitro* reverse transcription (Fig. 2). Presumably, the *in vitro* reaction is a good reflection of what happens in the cell, since it is possible to synthesize *in vitro* complete double-stranded DNA copies of the RNA genome that are infectious (100,249). Viral DNA synthesis *in vivo* takes place primarily during the first six hours after infection (342), in the cytoplasm of the cell (328,342). The transcription starts at about 100 nucleotides from the 5' end of the viral RNA where a primer-tRNA molecule is bound to the genome (cf. section 2.2.5.). The tRNA molecule serves as a primer for the synthesis of the (-) strand DNA. The first deoxynucleotides are covalently bound to this molecule (307,342), that in its turn is

removed at some later stage of the process from the newly synthesized DNA (101,193). The DNA synthesis proceeds to the 5' end of the RNA, jumps to the 3' end and reads further in 3'→5' direction (307,333,342). Recent results (115) incontrovertibly confirm earlier suggestions (e.g. 44,46,146) that the terminal redundant sequence (cf. section 2.2.5.) is used to elongate the DNA chain past the 5' end of the genome. The nascent DNA chain synthesized at the 5' end of the genome becomes displaced from the template (by the action of viral RNAse H, that is intrinsically associated with reverse transcriptase and degrades RNA in RNA.DNA hybrids; 49) and pairs with the repeated sequence at the 3' end of probably the same RNA molecule. The DNA, annealed to the 3' end of the RNA genome, serves as a primer for further elongation. So in this region of the proviral DNA only one copy of the 3',5' terminal redundant sequence is present (115). Reverse transcription proceeds to the 5' end of the viral RNA until it reaches the primer binding site region (101) and then "jumps" over to the 3' end of the (+) strand DNA (101,330).
The synthesis of the (+) strand DNA starts before completion of the (-) strand (342) and is directed (101,193) by the DNA-dependent DNA polymerase activity also intrinsically associated with the reverse transcriptase enzyme (106,257,308,333). The (+) strand DNA is produced in short pieces (342). The first piece that is synthesized has a length of 300 bases in the case of avian sarcoma virus (ASV) (330) and of 600 bases with murine leukemia virus (MuLV) (193). This piece contains sequences of both the 5' end and the 3' end of the genome (193,330) and therefore must be complementary to the 5' end of the (-) strand DNA. It has been shown that the complement of the first approximately 20 nucleotides of the tRNA primer - that apparently at this stage is still attached to the 5' end of the (-) strand - is present at the ultimate 3' end of this piece of (+) strand DNA (101).
The synthesis of the (-) strand DNA is completed by the transcription of this piece of (+) strand DNA (101,330), thus leading to a long repeat at the 3' end of a sequence already present at the 5'

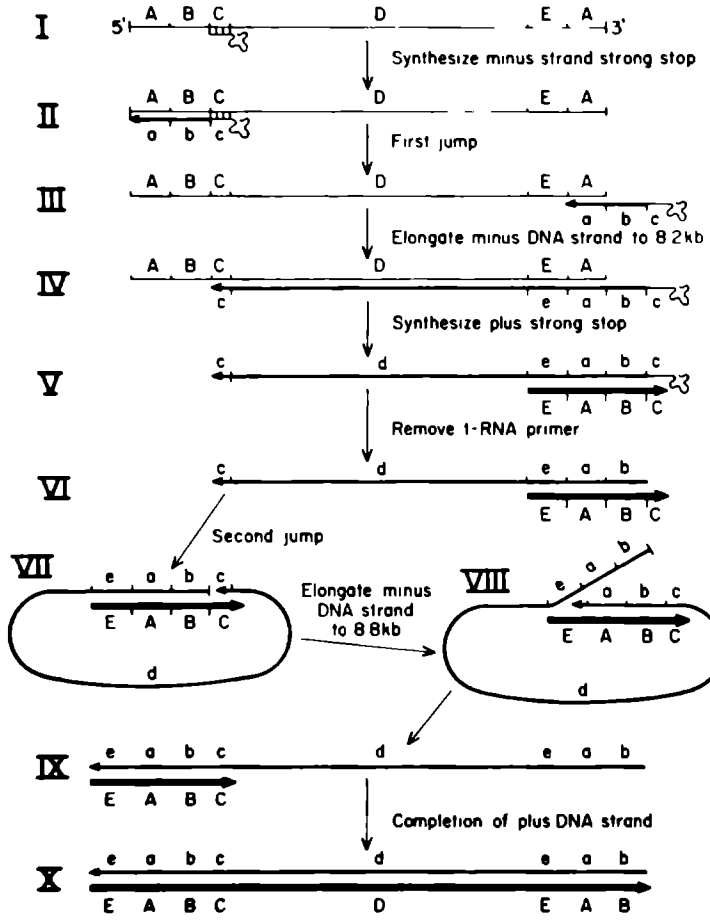

Figure 2. Model for reverse transcription of murine leukemia virus. This picture was reprinted from reference 101 with permission of the authors and the publisher. (Copyright held by Massachusetts Institute of Technology, Cambridge, Mass.).
Structure I represents one molecule of the dimeric RNA molecule of the virion. In II, minus-strand DNA synthesis, and in V, plus-strand DNA is initiated. Various regions - not shown on scale here - are represented by letters. Uppercase letters refer to sequences in the viral RNA molecule (plus-strand sequences), and lower-case letters refer to minus-strand sequences. The cap at the 5' end and the poly(A)-stretch at the 3' end of the viral RNA was omitted.

end of the (-) strand.

The pieces of (+) strand DNA complementing the whole (-) strand are ligated and a linear double-stranded DNA molecule is formed (100,101). Of course, in order to enable this formation, the genomic RNA and the primer tRNA must be removed first. Perhaps the viral RNAse H works in a concerted action with the reverse transcription activity of the same molecule and breaks down the RNA immediately after its complementary DNA is formed. The linear double-stranded DNA still contains the long terminal repeats of 600 bases in the case of MuLV (100), 300 bases in the case of ASV (127,268) and 1200 bases in the case of mouse mammary tumor virus (MuMTV; 269). This molecule moves to the nucleus, where it is converted into a closed double-stranded circular DNA molecule (127,241,268,269,270,342,355). Two kinds of circles are found. A minor form is coextensive with the linear molecule, but the predominant form of the circular DNA misses one of the terminal redundancies present in the linear molecule (100,127,268,269,355). The linear as well as the circular DNA is infective (342). It is believed that the circular form of the provirus is integrated into the cellular DNA, presumably just like the lambda phage is integrated into the bacterial genome (121), thus becoming an integral part of the host genome (327). The proviral DNA can be inserted at several, possibly specific, sites of the genome (60,133,156,158,292,321). The integrated DNA is coextensive with the linear and the larger form of circular unintegrated proviral DNA (133), so it contains the long terminal repeats. The second copy of the repeat is not derived from the cellular DNA (133), therefore, the most obvious candidate to provide this repeat would be the larger circular DNA molecule, although one could imagine a mechanism through which the second repeat would be generated (133). Therefore, the major form of circular unintegrated proviral DNA,that only contains one copy of the 3',5' repeat, is probably not infectious. Since this species is the most abundant form of circular proviral DNA, it is expected that circular proviral DNA is considerably less infective than linear proviral DNA.

In fact, this is what is found: circular proviral DNA has a five-fold lower infectivity than linear proviral DNA in transfection experiments (91).

Expression of the integrated proviral genome may be (51,52) or may not be (52,185) under control of adjacent (promoter) sites on the cellular DNA. Transcription into RNA is performed by the cellular RNA polymerase II (145,253). Within the nucleus, part of this RNA is spliced into subgenomic RNA (cf. section 2.4.4.5.). The RNA is transferred to the cytoplasm and can either act as messenger RNA or becomes integrated into the progeny virus. Messenger RNA and virion RNA have the same polarity. They presumably constitute separate pools (cf. section 2.4.3.).
Core plus inner coat proteins, reverse transcriptase and envelope proteins are derived from separate precursor polyproteins (cf. section 2.4.2.). The envelope precursor polyprotein is made on the rough endoplasmic reticulum and transported via the Golgi-apparatus to the plasma membrane where it is processed to the mature envelope proteins (117,346). Viral gp70 molecules are expressed on the cell surface (77,173,348,353). In some cases the envelope precursor polyprotein is present on the outer cell surface too (77). The precursor of the core plus inner coat proteins is presumably synthesized both on membrane-bound ribosomes and on free polysomes (271) or is first made in the cytoplasm and becomes very rapidly associated with endoplasmatic membranes (196). The precursor moves to the plasma membrane, but remains below the cell surface (175, 176,353). The core precursor polyproteins presumably associate with genomic RNA (143) - which is then still in 30-40S form (37, 165) - and with reverse transcriptase precursor molecules, establishing and immature core particle. The virus is released by budding of the viral core particle through the cellular membrane at sites where envelope proteins are arranged in proper order. During budding and directly after virus release the core precursor and the reverse transcriptase precursor molecules are cleaved into

the mature proteins p15, p12, p30, p10 and p75 (186,348,353,356), concomitant with a rapid morphological maturation of the virus that can be seen in the electromicroscope as a change from particles with an electron-lucent core center to particles with a condensed nucleoid (165,186,356). The major steps of the replication cycle are depicted in Fig. 3.

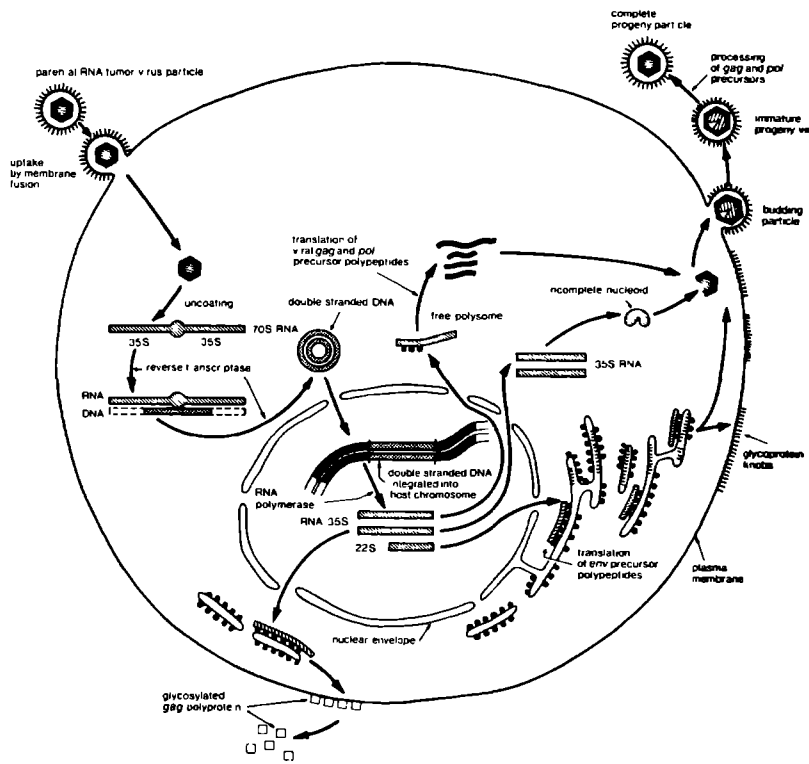

Figure 3. Schematic diagram of the major steps in the replication of murine leukemia virus.
(Modified after J.D. Watson, 1976, The molecular biology of the gene, 3rd. ed., p. 674. W.A. Benjamin Inc., Menlo Park, Cal.).

2.4. RETROVIRAL GENE EXPRESSION

2.4.1. THE VIRAL GENOME

The genome of non-defective RNA tumor viruses contains three or four known genes: gag, encoding the structural proteins of the viral core, pol, encoding the viral reverse transcriptase, env, encoding the (glyco)-proteins of the viral envelope and, in some cases, onc, that is responsible for oncogenic cell transformation (8).

The genome order has been established for the avian sarcoma virus (ASV) (147,337,338,339) and runs 5'-gag-pol-env-src-poly(A)-3' (src denoting that this virus causes sarcomas). Between the src gene and the poly(A) stretch of ASV a region of about 1000 nucleotides is located, the so-called "C" or common region, that is conserved in all variants, mutants and recombinants of ASV (306). It was never formally proven (because of the lack of suitable mutants) but there is ample indirect evidence to believe that the gene order for the mammalian leukemia and mammary tumor viruses is the same, although most probably these viruses lack a separate onc gene.

2.4.2. PRIMARY GENE PRODUCTS

The primary gene products of the non-defective oncoviruses are known. This subject has been described in detail in several recent reviews (67,142,298). The internal core proteins, the envelope proteins and the reverse transcriptase are synthesized in the form of precursor polyproteins that are processed and split into the mature virus proteins by cellular enzymes or by enzymatic activities of the viral proteins themselves (336). In the case of murine leukemia virus the precursor of the core proteins, $Pr65^{gag}$,

has a molecular weight of 65,000. $Pr65^{gag}$ is phosphorylated (260) and is cleaved through several intermediates into the mature core proteins p30, p15, p12 and p10 (4,144,272,324). By analyzing the gag products formed by defective recombinant viruses that lack part of the gag gene (10) and the gag processing intermediates found in cells infected with temperature sensitive mutants each blocked at different stages of the gag precursor cleavage (238) the subgenome order of the gag gene was established as 5'-p15-p12-p30-p10-3'. This conclusion was confirmed by the results obtained by studying the premature termination products formed during translation of viral RNA in cell-free systems (203; see below). In addition to $Pr65^{gag}$ another gag polyprotein of 75,000-80,000 daltons can be detected that is rapidly metabolized. This protein was regarded as the immediate precursor of $Pr65^{gag}$ (4,144,272,324) but recently it was recognized that $Pr65^{gag}$ and the 75,000-80,000 gag protein presumably are translated from two separate initiation sites on the viral RNA (66,228). It may even be possible that both proteins are made on separate messengers. The mRNA for $Pr65^{gag}$ could miss the sequence coding for the peptide (259) that the 75,000-80,000 protein contains in addition to the $Pr65^{gag}$ sequence.
Alternatively it is conceivable that one gag-mRNA originates from the other one by a small splice that creates a new gag initiation site.
The 75,000-80,000 protein was shown to be moderately glycosylated (66,260). The unglycosylated form of $gP80^{gag}$ has a molecular weight of 72,000 (66,260). On further glycosylation $gP80^{gag}$ changes into $gP95^{gag}$. In some cells $gP95^{gag}$ can be processed to $gP85^{gag}$ (66,71, 175,176,260). These glycosylated gag polyproteins contain all sequences of p15, p12, p30 and p10 (175) but are hard to detect in some systems or when not labeled appropriately (i.e. with mannose). The proteins $gP95^{gag}$ and $gP85^{gag}$ are expressed on the cell surface of at least some cell lines (174,175,176,320) and were shown (176) to represent the Gross cell surface antigen (GCSA). The envelope proteins gp70, p15E and p12E have a glycoprotein of

82,000 ($gPr82^{env}$) as their common precursor (144,152,206,272,324). The subgenome order of the *env* gene is: 5'-gp70-p15E-3' (152). The envelope protein p12E is a processing product of p15E (152,206, 324). The precursor of the reverse transcriptase has a molecular weight of 180,000 to 200,000 and contains both *gag* and *pol* sequences ($Pr180-200^{gag-pol}$; in fact two molecules with slightly different molecular weights are synthesized; 144,163,348). In the first processing step the *gag-pol* precursors loose their *gag* sequences and through several intermediates the mature polymerase (molecular weight 70,000 to 80,000) is formed (144,163).
Only on the avian sarcoma virus genome a separate *onc* gene is clearly identified. The *src*-gene of the virus has a 60,000 daltons phosphoprotein (pp60) with protein kinase activity as its product (33,48,180).

2.4.3. VIRUS-SPECIFIC RNA CONTENT OF INFECTED CELLS

Several investigators have studied the retrovirus-specific RNA content of infected cells using DNA complementary to viral genomic RNA as a probe. Depending on cell line and virus used, the amount of virus-specific RNA in permanently infected normal host cells ranges from 0.02 to 1 % of the total RNA content (32,65,78,113,177, 245,316,331,344).
Two to 15 % of the virus-specific RNA is found in the nucleus (113, 177,245), coinciding with the total RNA distribution pattern. While 70 to 80 % of the total cytoplasmic RNA is found in polyribosomes only 40 to 60 % of the cytoplasmic virus-specific RNA is polyribosome-bound (65,331). The free cytoplasmic virus-specific RNA has a much faster turnover than the ribosome-bound virus-specific RNA and is thought to represent RNA to be encapsidated in virions (179). Membrane-bound polyribosomes are enriched for the virus-specific RNA since they contain 50 % of the polyribosomal virus-specific RNA (78,98) compared to only 20 % of the total

polysomal RNA in mouse fibroblasts (78). Reported ratios of poly(A)$^+$ RNA to total cytoplasmic virus-specific RNA range between 0.2 and more than 0.9 (32,65,107,222,245,331,344), depending on the system studied or the method used.

2.4.4. SIZE-CLASSES OF VIRUS-SPECIFIC CELLULAR RNA

Cytoplasmic virus-specific RNA is present in different size classes. Cells productively infected with avian sarcoma virus contain three classes of viral RNA (119,222,344): 38S (3.3 x 10^6 molecular weight), 28S (1.8 x 10^6) and 22S (1.2 x 10^6).

In addition to these three major size classes Krzyzek et al. (167) found a minor class of 14S (0.4 x 10^6) RNA. This 14S RNA was also seen by Brugge et al. (32) but this group did not detect a 28S virus-specific RNA.

Weiss et al. (344) and Hayward (119) showed by hybridization with gene-specific probes that the 38S viral mRNA encodes all four genes and is probably identical to the viral genome. The 28S RNA encodes the 3' half of the viral genome: 5'-env-src-C'-poly(A)-3'. The 22S viral mRNA comprises the sequence 5'-src-C'-poly(A)-3'. Cells infected with deletion mutants that miss either the src or the env gene have only two size classes of viral RNA: 35S (3 x 10^6) which comprises the same sequence as the genomic RNA of the deletion mutants, and 22S (1.2 x 10^6) with the sequences 5'-env-C'-poly(A)-3' or 5'-src-C'-poly(A)-3', respectively (119,344).

Murine leukemia virus-infected cells contain at least two size classes of virus-specific mRNA (78,80,98,315): 35S (3 x 10^6 molecular weight) and 22S (1.2 x 10^6). In our laboratory 14S (0.4 x 10^6) viral RNA (99,123,325) was also detected, and others (315) found heterogeneous virus-specific RNA species between 10 and 18S. One should note, however, that at the time these analyses were performed well-defined copy-DNAs were not available. Therefore, the 14S could originate from the infecting virus as well as represent (deleted?)

endogenous viral sequences or even normal cellular sequences. Fan and Verma (80) showed that the subgenomic 22S mRNA is derived from the 3' half of the MuLV genome since the 22S RNA did not hybridize with a copy-DNA made specific for the gag-pol sequences ($cDNA_{gag-pol}$). Haseltine and Baltimore (113) and Fan (79) studied the viral RNA content of the nuclei of MuLV-infected cells. In both studies 35S as well as 22S virus-specific RNA was found in the nucleus. This was in agreement with studies on mouse mammary tumor virus-infected cells by Robertson and Varmus (245) that also showed that the same virus-specific RNA size classes were present in the nucleus as were present in the cytoplasm (see below).
Particularly Fan (79) showed in pulse-label experiments that no larger precursor to the genome size viral RNA could be detected. In addition he found that the 22S RNA is presumably not a direct transcription product but is derived from 35S RNA in the nucleus.

In Moloney murine sarcoma virus-transformed, non-producing cells only genome-size (30S) RNA could be detected with a DNA-probe complementary to the parental leukemia virus (315,316), but it is conceivable that these cells contained subgenomic virus-specific RNA that consisted of sarcoma virus-specific sequences only. Very recently a subgenomic viral RNA (22S) was found in a Moloney murine sarcoma virus producing cell line that overproduced the sarcoma virus relative to the helper leukemia virus (62). This subgenomic viral RNA had lost those Mo-MuLV -specific gag sequences that are still present in the Mo-MuSV genome (cf. section 2.5.), but had retained the -incomplete - parental virus-specific env sequences of the 30S Mo-MuSV RNA. One should note, however, that it is not certain that producing and non-producing murine sarcoma virus-infected cells express their sarcoma provirus in the same way.

The mouse mammary tumor virus (MuMTV) is a B-type virus and it has main characteristics in common with the C-type retroviruses.

Robertson and Varmus (245) showed that treatment of MuMTV infected cells with dexamethasone stimulated the viral RNA expression in the cell severalfold. These authors as well as Sen et al. (266) found three size classes of virus-specific intracellular RNA: 35S (3.1×10^6), 24S (1.5×10^6) and 14S (0.4×10^6). The 14S RNA was hard to detect with a cDNA against the total MuMTV genome but could readily be seen with a cDNA made specific for the 3' end of the genome ($cDNA_3'$). Groner et al. (107) did not detect the 14S mRNA, although they used the same cells and $cDNA_3'$. On the other hand they found in addition to 35S and 24S virus-specific RNA a 33S (2.9×10^6) viral RNA that only could be detected in poly(A)$^+$ cytoplasmic RNA but lacked the information of the 3' end of the MuMTV genome.

2.4.5. SPLICING

It was shown by several groups that the subgenomic virus-specific RNAs contained a leader sequence of at least 150 nucleotides derived from the 5' end of the genomic RNA. In ASV infected cells both 28S and 22S RNA (possibly 14S RNA too; 167) possessed this 5' sequence of 38S RNA (54,167,191,344). Mellon and Duesberg (191) and Cordell et al. (54) showed that the three major size-classes of viral RNA from ASV infected cells had the same cap structure. In MuLV (80) and MuMTV (245) infected cells 35S and 22-24S RNA hybridized with $cDNA_5'$ but 14S RNA did not.
Also electron-microscopic analyses of hybrids between subgenomic MuLV RNA and genome-length cDNA showed a common 5' sequence for 35S and 22S RNA that was not present in 14S RNA (220,250).
Since the 22S virus-specific RNA can be found in the nucleus, while virus-specific nuclear RNA larger than genome size is not detectable (79,113,245), it is assumed that the 22S RNA is generated in the nucleus from the 35S RNA by splicing out the sequences between the common 5' leader region and the 3' half of the

genome. Stacey and Hanafusa (288) showed directly that viral genomic RNA can be spliced in the nucleus into active env-mRNA. They microinjected 35S RNA from Rous-associated virus-2 (RAV-2, an avian leukosis virus, and, therefore, lacking the src gene and not able to transform chicken fibroblasts) into nuclei of cells infected with the env deficient Bryan high titer strain of Rous sarcoma virus (BH-RSV). The RAV-2 RNA appeared to complement for the deficient env function of BH-RSV and, therefore, must have been spliced into 22S env-mRNA. The possibility that in these experiments the injected 35S RNA was reversely transcribed by the BH-RSV polymerase present in the cells and that the env-proteins were products of 22S mRNA transcribed from newly integrated RAV-2 proviral DNA, could be excluded for kinetical reasons and by a parallel experiment in which 35S RAV-2 RNA was injected into the nuclei of chick embryo fibroblasts which expressed no viral functions, that also resulted in the production of infectious (RAV-2) viruses.

2.4.6. TRANSLATION PRODUCTS OF VIRION RNA

It was shown by several groups that the 35S RNA from virions only directs the synthesis of the gag precursor proteins and - as was shown more recently - of the gag-pol precursor (15S,190,201,202, 210,223,224,228,255,266,345). Philipson et al. (228) were able to augment the production of the MuLV gag-pol precursor by 35S viral RNA with the addition of yeast suppressor tRNA to the cell-free system. Therefore they proposed a "read-through" mechanism for the synthesis of the precursor of reverse transcriptase: at relative low frequencies the gag termination codon - at least the termination codon of the mRNA for $gP80^{gag}$ - would be suppressed leading to the synthesis of a gag-pol precursor protein. These ideas were not substantiated by the results of Weiss et al. (345), who could not find any effect of suppressor tRNAs on the synthesis of the

ASV gag-pol precursor. They suggested that the pol-mRNA has the same size and the same genetic content as the gag-mRNA, except for a small sequence spliced out in one of the mRNAs, leading to the presence of a termination codon in the gag-mRNA and its absence in the pol-mRNA. According to recent findings of Hizi et al. (125) and Kopchick et al. (164) the gag termination codon would be situated within the 5' end of the pol gene, since the gag precursor protein and the mature reverse transcriptase shared a short sequence.

Translation of virion RNA smaller than genome size did not seem to yield any virion protein (155,189,210,224,255,266) but recently more sensitive systems showed that virions contain low levels of subgenomic viral RNAs that actively direct the synthesis of viral proteins. The src gene product of RSV was recognized in translations of viral RNA of about 22S in nuclease-treated cell-free systems (16,150,234). It was made in vitro as a precursor protein of 62,000 molecular weight, that was processed to a 60,000 protein after the addition of dog pancreas membranes to the cell-free system (149), suggesting the presence of a "signal peptide" (28) in the primary transcript. By tryptic peptide mapping the 60,000 in vitro product was shown to be essentially identical to the product found in vivo (234,267). It is uncertain whether the viral RNA synthesizing the src protein is a product of 35S RNA cleavage in the virion or a cellular messenger RNA encapsulated by the virus particle during the budding process.

2.4.7. TRANSLATION PRODUCTS OF CELLULAR VIRUS-SPECIFIC mRNAs

Gielkens and Van Zaane et al. translated size-fractionated mRNAs from MuLV-infected cells in reticulocyte cell-free systems (99) and in oocytes of the frog Xenopus laevis (325) and showed that 35S mRNA was synthesizing gag proteins and 22S mRNA produced env proteins. Mueller-Lantzsch and Fan (200) at the same time found

that only polyribosomes containing 35S virus-specific mRNA could be immunoprecipitated with anti-p30 antibodies. Mature reverse transcriptase can be synthesized in oocytes by injection of 35S mRNA from infected cells (chapter 4 of this thesis). These results are confirmed by those of Murphy and Arlinghaus (204) who translated cellular messenger RNAs in a nuclease-treated mouse cell extract. They showed that 35S mRNA synthesized the gag-pol precursor, 25-35S mRNA the gag precursor proteins and mRNA around 22S the non-glycosylated env precursor. Translation products were identified by peptide mapping. Similar results were obtained by Sen et al. (266) with mRNA from MuMTV infected cells.
In the avian system Pawson et al. (225) found the 38S RSV-specific mRNA to synthesize the gag precursor protein in a cell-free system derived from mouse L cells, while 20-28S mRNA generated the non-glycosylated form of the envelope precursor. Stacey et al. (287) showed by microinjection of 22S mRNA from RAV-2 infected cells into the cytoplasm of BH-RSV infected cells (cf. preceding section) that avian leukosis viral cytoplasmic mRNA of 22S size directs the synthesis of envelope proteins, since they could detect focus-forming infectious virus 3 - 10 h after injection.

Several groups (98,99,325) found virus-specific cellular RNA in the 14S region that lacked the 5' sequence which larger subgenomic viral RNAs have in common with the genomic RNA (cf. section 2.4.5.). RNA of this size has a maximal coding capacity of about 40,000 daltons of protein. No product of the 3' region is known and 3' region-specific products - if existing - may not be identified unless virus-specific mRNAs are isolated free from contaminating cellular messengers. In suggestive, though inconclusive experiments we found products of 14S mRNA, both in the cell-free system and in Xenopus oocytes, that were immunoprecipitable with antiserum against whole disrupted Rauscher murine leukemia virus but not with monospecific antisera (99,325).
In oocytes this 14S mRNA product has a molecular weight of about

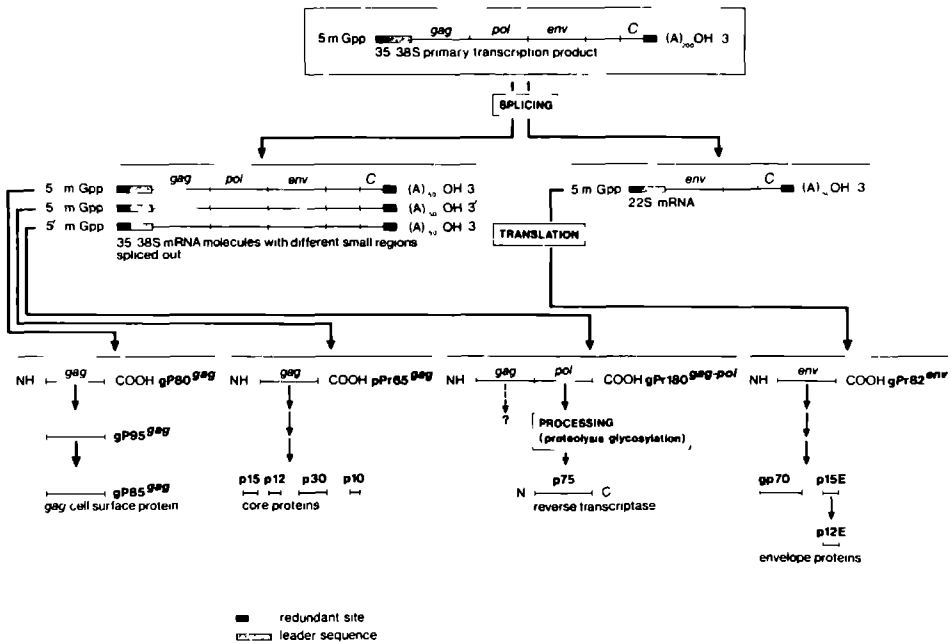

Figure 4. Gene expression of murine leukemia virus.
(The exact nature of the primary transcription product and the gag and gag-pol mRNAs is still uncertain).

37,000 (325). Since it is known that virus-coded non-structural proteins different from the reverse transcriptase can be encapsidated into the virions (157,236,273) it is conceivable that the virus preparation we used for raising the anti-R-MuLV serum contained a product coded for by the ultimate 3' region of the viral genome. We could then detect this protein among the translation products of 14S mRNA because the anti-R-MuLV serum contained antibody activity against it. Alternatively the antiserum contained antibodies directed against some cellular protein contaminating the virus. This latter possibility seems to be true, since in later experiments we were able to precipitate the 37,000 molecular weight protein also from the translation products of 14S mRNA from uninfected cells (chapter 5. of this thesis).

2.4.8. CELLULAR mRNAs ENCAPSIDATED BY VIRUS PARTICLES

Not only cellular proteins may "slip" into the virus particle but also cellular non-genomic virus-specific mRNAs may become part of the virion.
If $Pr65^{gag}$, $gP75^{gag}$ and $Pr180-200^{gag-pol}$ are indeed synthesized on at least three separate mRNAs (cf. sections 2.4., 2.6.), two of these mRNAs would be the first candidates for non-genomic mRNAs in the virion. These two hypothetical non-genomic viral RNAs should not be distinguishable in size from the genomic RNA and must be encapsidated by the virion efficiently, since 35S viral RNA generates $P75^{gag}$ (the unglycosylated form of $gP80^{gag}$), $Pr65^{gag}$ and $Pr180-200^{gag}$ in about the same proportions *in vitro* as they are made *in vivo*. The 22S RNA present in ASV particles that synthesizes the *src* gene product would be the third example of a virus-specific cellular mRNA present in the virion, although Purchio et al. (234) showed that partial ribonuclease digestion of 35S viral RNA produced RNA that was able to synthesize *src* products too.

Most groups did not detect any env mRNA activity in virion RNA but Philipson et al. (228) showed that their MuLV RNA synthesized the env precursor at a very low level. Moreover, by injection of 22S RAV-2 virion RNA in BH-RSV infected cells which gave rise to the production of focus-forming infectious virus, Stacey (289) showed the encapsidation of env mRNA by the virion. In this case RNAse T_1-generated fragments of 35S viral RNA were unable to promote the release of infectious virus.

So it seems that virions can contain non-genomic virus-specific RNA somehow bound to the 50-70S viral RNA complex. In fact, it appears that viral RNA is contaminated at low levels with many species of cellular mRNA (16,149,150,203,234,266,267; C. Saris, personal communication).

2.5. RECOMBINANT VIRUSES

RNA tumor viruses can easily recombine: either with each other, when cells are coinfected with different virus strains (340) or with a part of the host genome. In the latter case, either an endogenous viral genome or - though very infrequently - an other, cellular, sequence may be involved. This distinction becomes rather arbitrary, if one assumes that all viral sequences are originally cell-derived ("protovirus" hypothesis of Temin; 309). Differences may exist, however, in the evolutionary stability of these host cell derived sequences. It is not known whether recombination takes place at the DNA level or at the RNA level. Recombination at the RNA level may be promoted by the formation of mixed dimers of genomic RNA, that presumably readily occurs because of the appearance of heterozygous virus particles at relatively high frequency in mixed infections (188,326,343).

The src-gene of avian sarcoma virus (ASV) and the sarc-regions[1] of all known mammalian sarcoma virus strains are host cell-derived (244,326,334,341) and it is believed that these viruses originate from a recombination between an exogenous leukemia virus and some host cell sequence (244,326,334,341). Viruses having these cell-derived sequences integrated into their genome are able to transform fibroblasts in cell culture and to cause sarcomas in vivo. Their parental leukemia viruses can not transform fibroblasts. They induce haemopoietic malignancies. All sarcoma viruses contain the 5' end and the 3' end of their parental leukemia virus (326; section 2.2.4.). An important distinction between ASV and mammalian

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1) The use of the term "src" should be restricted to denote a genetic function. The term "sarc" is used to indicate the cell-derived or sarcoma virus-specific sequences in the genome of sarcoma viruses (326).

fibroblast-transforming viruses is the dependence of the latter ones on helper virus activity for replication (cf. section 2.1.). In mammalian sarcoma viruses the sarc-region replaces structural genes of the parental virus (326), while in ASV the src-gene is present in addition to the structural genes derived from the parental leukosis virus. However, other avian viruses resemble the mammalian sarcoma viruses in this respect. Avian myelocytomastosis virus-29 (MC29) and Mill Hill virus-2 (MH2) are replication-defective viruses, which lack the src-gene of ASV but contain a mutually related cellular sequence integrated in the middle of their genome, replacing the 5' end of the env-gene, the whole pol-gene and the 3' end of the gag-gene of the parental avian leukosis virus (17,64, 128,129,130). Other examples are the avian erythroblastosis virus (AEV), the myelocytomastosis-type viruses CMII and OK10 and the myeloblastosis-type viruses AMV and E26 (170,251). These replication-defective avian viruses cause acute leukemias, lymphomas, sarcomas and carcinomas in chickens (104). The host cell sequences present in ASV and in MuSV have no homology to known viral sequences and are evolutionary much more stable than virus-specific sequences of either exogenous or endogenous origin (20,90,92,169,207,281,286, 293,310,326). Therefore, it is presumed that these src-genes or sarc-sequences code for important cellular functions. The cell-derived sarc sequences are usually not related to each other. One exception form the rat cellular sequences present in Kirsten and Harvey murine sarcoma virus (Ki-MuSV and Ha-MuSV) (277,326). Furthermore, the avian replication defective viruses can be divided in three groups on the basis of the homology of their cell-derived sequences (251).

A number of murine viruses exist which have similar properties as the sarcoma viruses but which do not induce sarcomas. They cause acute haemopoietic malignancies, but are replication-defective and are able to transform cells in vitro. Examples are the Friend strain of spleen focus-forming virus (F-SFFV) that causes splenomegaly and transforms certain bone marrow cells (326), Abelson murine leukemia

virus (A-MuLV) that induces lymphomas (326) and transforms pre-B-lymphocytes as well as fibroblasts (284), and replication-defective strains of mink cell focus-forming (MCF) virus which cause lymphomas and transform mink cells (111,335). MCF-viruses have been isolated from a number of mouse strains that were infected with different exogenous viruses or that expressed endogenous ecotropic virus (40, 111,254,335). They emerge concomitantly with a rapid tumor formation. When injected in newborn mice, they induce tumors considerably more rapidly than ecotropic leukemia viruses do. Therefore, they are presumed to play a central role in tumor formation and are, perhaps, the primary tumor-causing agents. They have an amphotropic host range and derive their name from the cytopathogenic effect they exert on mink cells. They have been shown to arise by recombination in the env-gene of the ecotropic virus with an endogenous xenotropic virus (40,69,246,276). Another example of an amphotropic virus that causes rapidly evolving lymphomas and that is an env-gene recombinant between ecotropic and xenotropic virus, is the HIX-virus, isolated from a Moloney-MuLV stock (74,75,88).

Finally, F-SFFV is an env-gene recombinant between ecotropic and xenotropic virus. The xenotropic env-regions of F-SFFV and MCF are related (252,276). They both express mutually related envelope glycoproteins (62,252). It is proposed that all highly oncogenic variants of murine ecotropic viruses are derived by genetic recombination leading to the acquisition of a common class of xenotropic virus-related envelope glycoprotein determinants, which are presumably required, but not necessarily sufficient for oncogenicity (60).

The env-proteins of MCF-viruses present on the cell surface do not only contain ecotropic and xenotropic determinants but also have MCF-virus -specific epitopes (43). An interesting hypothesis regarding the mechanism of leukemia-induction by MCF-viruses has been put forward by McGrath and Weissman (190). These authors showed the gradual emergence of specific MCF-virus receptors on preleukemic and leukemic thymocytes of AKR mice. The presence of

Table 2.

Gene expression of some recombinant RNA tumor viruses.

Species	Virus	Expression of						P_p (MW)	NR (MW)
		gag				pol	env		
		mammalian:	p15	p12	p30	p10	p75	gp70		
		avian:	p19	p12	p27	p15	p110/70	gp85/35		
mouse	Mo-MuSV		±	±	±	-	-	±	58,70	
	BALB-MuSV		+	+	±	±	?	-		
	F-SFFV[a]		+	+	±	-	?	gp55	120	
	A-MuLV		+	+	-	-	-	-	120	(90)
	MCF,HIX[a]		+	+	±	±	±	±	100[d]	
	B-MuX		+	+	+	+	+	+	-	
rat	Ki-MuSV, Ha-MuSV[b]		-	-	-	-	?	-	-	21
cat	FeSV		+	+	±	-	-	-	85-120	(60)
woolly monkey	SiSV		±	±	±	-	?	-	-	
chicken	ASV		+	+	+	+	+	+	-	60
	AMV, E26		?	?	?	?	?	?	?	
	MC29, MH2, CMII, CK10[c]		+	+	+/-	-	-	-	90-110	
	AEV		+	+	+/-	-	-	-	75	

Legend:

+ : expressed
- : not-expressed
± : expression dependent on virus strain; as a general rule, if a particular protein is not expressed, all virion proteins coded for more 3' terminally on the parental viral genome are not expressed either, with the exception of the viral glycoproteins of the S^+L^- strain of Mo-MuSV and of F-SFFV which are still expressed, though of the gag gene only p15 and p12 are expressed.
+/- : partially expressed.
P_p : p15-p12-(p30)-X polyprotein; molecular weights in thousands
NR : translation product of cellular sequences in the recombinant genome, not related to any known protein; molecular weight in thousands
a : F-SFFV and MCF express related env proteins
b : Ki-MuSV and Ha-MuSV contain related rat cellular sequences and produce a similar sarc-product
c : MC29 and MH2 contain related onc sequences and produce related polyproteins
d : only present in some replication-deficient virus strains

This table is compiled from the data and references cited in the text.

these receptors correlated with the tumorigenicity of the pre-leukemic thymocytes. They suggested that recognition by these receptors of MCF-viral antigens would induce a mitogenic stimulus to the thymocytes, similar to antigen-induced proliferation of antigen-reactive T-lymphocytes. This quality should be attributed to the specific determinants on MCF-virus envelope proteins. Since in lymphoid organs (pre)leukemic lymphocytes are always surrounded by other lymphocytes expressing MCF-viral envelope antigens, they are continuously stimulated to proliferate, thus leading to the formation of a lymphoma. This model explains the absence of any indication for the presence of a separate onc-gene in MuLV, since in this concept the MCF-viral envelope antigens would be oncogene products.

F-SFFV and some strains of MCF are replication-defective and must have deletions or mutations, that occured during or after the recombination event. This might well have happened, since replicating viruses show a high frequency of aberrant expression caused by this kind of events (274). Likewise, it has been shown that isolates of simian sarcoma virus (SiSV; also called: woolly monkey sarcoma virus, WoSV) that differed in the expression of helper leukemia virus-specific gag-proteins, did not differ in the extent of their helper virus-derived gag-gene region (243). It was assumed that small deletions or mutations caused premature termination of translation or the production of immunologically non-reactive proteins (243), possibly because of a frame shift.

Gene expression of mammalian sarcoma viruses and other replication-defective viruses has been reviewed recently (326). In general, sarcoma viruses do not express envelope proteins (with some exceptions: 24,63,88,237,252) and reverse transcriptase. The regions of the leukemia virus genome encoding these proteins were lost during recombination and are replaced by cellular sequences. Often part of the helper virus-specific gag-gene -products are still expressed by the recombinant virus. Apparently, the chance of

recombination decreases towards the 5' end of the leukemia virus genome, since the probability of the expression of a gag subgene by the recombinant virus decreases in 5'→3' direction of the gag-gene (323,326). Products of the sarc-region of Moloney murine sarcoma virus (Mo-MuSV) are not yet known. Recently a 21,000 molecular weight phosphoprotein was identified as the product of part of the rat cellular sequences present in Ha-MuSV and Ki-MuSV (275,278). The src-gene product of ASV was discussed before (section 2.4.2.). A-MuLV (236,239,347), F-SFFV (11), a replication-defective strain of MCF (254), particular strains of Mo-MuSV (215) and a particular strain of feline sarcoma virus (FeSV; 233,297) synthesize polyproteins of 60,000-120,000 molecular weight, that contain helper-specific p15 and p12 and a smaller or larger part of p30 covalently bound to different non-structural proteins that may be responsible for maintenance of the transformed phenotype of cells infected by these viruses. The avian viruses AEV, MC29, CMII and MH2 produce similar polyproteins (118,128,129,192,251). Moreover, the non-structural component of the FeSV polyprotein is cleaved off, transported to the cell membrane and expressed as a cell surface antigen (feline oncornavirus-associated membrane antigen, FOCMA), that acts as a target for natural immunosurveillance against tumor development in the cat (70,297; cf. next section).

In summary one may say that recombination readily occurs with RNA tumor viruses, giving rise more or less accidentally to variants with new biological properties. For some of these recombinations it is thought that they form necessary steps in the process of leukemogenesis. Variants that result from this kind of recombinations will be positively selected by the organism.

2.6. VIRUS-RELATED CELL SURFACE ANTIGENS

A number of RNA tumor viruses are able to induce neoantigens on the cells in which they are present (14,168,171,183). All available evidence supports the notion that these neoantigens are virus-coded (14,168,183). They fall apart in three categories: 1. viral envelope proteins, 2. glycosylated gag polyproteins and 3. polyproteins consisting of the viral gag proteins p15 and p12 covalently bound to a non-structural, virus-coded protein.

Viral envelope glycoproteins are expressed on all virus-infected cells (41,135,262). Moreover, they are present on normal, non-virus producing mouse lymphocytes, presumably as the consequence of the partial expression of an endogenous virus (42,178,198,318). Especially the expression of xenotropic viral proteins in normal tissues is widespread in inbred mouse strains (124,198). Examples of surface antigens (CSAs) that were recognized as gp70-related proteins are: the FMR-antigen (a subgroup-specific CSA present on cells infected with the Friend, Moloney or Rauscher strain of MuLV; 209,213), the G_{IX}-antigen (a CSA present on Gross-MuLV induced leukemias; 183,211,317), the G_{RADA1}-antigen (defined by naturally occuring antibodies directed predominantly against N-ecotropic viruses; 212), the G_{ERLD}-antigen (defined by natural type-specific antibodies against xenotropic virus; 300), the G_{AKSL2}-antigen (defined by natural antibodies specific for leukemia-accellerating MCF-type viruses; 300) and the mutually related neoantigens found on F-SFFV and MCF-virus infected cells (103).
The antigens G_{IX}, G_{RADA1}, G_{ERLD} and G_{AKSL2} do not only occur on virus-infected cells, but they are also present on normal, non-virus -producing thymocytes. For instance, G_{IX} occurs on normal thymocytes and leukemic cells of the high leukemia incidence, virus producing AKR mouse strain, as well as on the thymocytes of low leukemia incidence, apparently virus-free mouse strains (e.g.

strain 129) (183,211,317,318). G_{IX} functions as a differentiation antigen for thymocytes in normal mice: its expression increases as the thymus cells mature (214).

According to some investigators viral envelope proteins of certain murine leukemia viruses are associated with histocompatibility antigens on the cell surface (7,35,120). Moreover, the histocompatibility antigens were found in released virions, still associated with envelope proteins (7,36). These results should be considered with care, however, since anti -H-2 sera can contain anti-gp70 antibodies (89), which are present in most mouse sera (137,139,162, 208,295). In one of the experiments, though, the association of FeLV envelope antigens with HLA antigens on human, FeLV-infected cell lines and in released virions was shown (7). It does not seem likely that human anti-HLA sera contain anti-FeLV antibodies. Other investigators failed to detect any association between mouse H-2 and murine leukemia virus proteins (3). Yet, the association between (subclasses of) histocompatibility antigens and (subclasses of) viral envelope proteins would support the "altered self" model (though certainly not excluding the "dual recognition" model) for H-2 restricted target cell recognition in antigen-directed T-cell cytotoxicity, originally described by Doherty and Zinkernagel (357). Equally relevant, in this context, is the observation that anti-serum against xenotropic virus (induced from BALB/c lymphocytes by a B-cell mitogen) could suppress the humoral immune response against a non-viral antigen by BALB/c mice *in vivo* as well as *in vitro* (89). This result would suggest that viral envelope antigens on the cell surface of immunoreactive cells might be involved in a more general way in the immune response (recognition and interaction of immunoreactive cells; *e.g.* T-B cell interaction).

In most immunological studies the exact nature of the gp70-related CSA was not ascertained. It was shown recently, however, that ecotropic and xenotropic viruses only induce the expression of the mature gp70 molecule on the cell surface, whereas MCF-virus -infected cells also express the *env*-precursor protein externally

(77).

Glycosylated gag polyproteins (apparent molecular weight about 95,000; cf. section 2.4.2.) have now been found on the surface of cells infected with Gross-MuLV (this antigen was already known as GCSA, Gross cell surface antigen; 174,175,285,319,320), Rauscher-MuLV (260), Friend-MuLV (50,71) and it is probably present on the surface of Moloney-MuLV -infected cells too (these cells were shown to contain the gag polyprotein, but its location was not determined; 66). In some cases a second glycosylated gag polyprotein with molecular weight of 85,000 (cf. section 2.4.2. and Fig. 3) was found (71,174,175,285,319,320). No studies are available on the presence of this gag polyprotein on cells producing endogenous virus or on non-virus -producing cells. Antigens related to p30 are present, however, in tissues and sera of numerous mouse strains (124).

Polyproteins containing gag p15 and p12 (plus a varying part of p30) covalently bound to a non-structural protein sequence are synthesized by a number of replication-defective, highly oncogenic viruses (cf. section 2.5.). The non-structural protein that is cleaved off from the polyprotein synthesized by FeSV, can be precipitated by an antiserum against the feline oncornavirus-associated cell membrane antigen (FOCMA) (297). Remarkably, this CSA can be found either on FeSV-induced tumors and FeSV-transformed fibroblasts or on FeLV-induced lymphomas, but not on FeLV-infected fibroblasts (70,168).
The polyprotein synthesized by Abelson-MuLV is processed similarly (at least in some cells; 236,239,347) and the non-structural protein is expressed on the outer surface of the infected cells (350). The relation of this CSA, that has a non-identical but cross-reacting counterpart in normal lymphoid cells (309), to the A-MuLV -related differentiation alloantigen found on A-MuLV -infected cells and on haemopoietic cells (bone marrow, spleen,

Table 3 Virus-related cell surface antigens.

Class	Virus-specificity	Antigen	Presence of antigen: After exogenous infection	Presence of antigen: High-leukemic animals: tumors	Presence of antigen: High-leukemic animals: normal tissue	Presence of antigen: Low-leukemic animals	Presence of antibody: After exogenous infection	Presence of antibody: High-leukemic animals	Presence of antibody: Low-leukemic animals	Role in immune response
Viral envelope glycoproteins (gp70)	ecotropic	G_{IX}	+	+	+	+			+	diff. antigen
		G_{RADA1}	+	+	+	−		−	+	
		FMR	+	−	−	−	+	−	−	
		MCSA	+							
	xenotropic	$G_{ERLD'}$ XenCSA			+	+		+	+	diff. antigen
	amphotropic	G_{AKSI2}	+	+	+	−		+	−	diff. antigen?
		MCF-CSA	+							
		F-SFFV-CSA	+							
Glycosylated gag polyproteins	ecotropic	GCSA	+	+	−	−				
		Rauscher-CSA	+							
		Friend-CSA	+							
		Moloney-CSA	+							
	xenotropic	(p30-related)			+	+		+	+	
polyproteins p15-p12-(p30)-X		Abelson-CSA	+							diff. antigen?
		FOCMA	+	present in tumor-bearing cats			naturally present in cats			tumor rejection

This tabel is composed of the data and references cited in chapter 2.6.

fetal liver) of young BALB/c mice, but not on adult liver, thymus or peripheral blood lymphocytes, either from BALB/c or from other mouse strains (242), is not yet clear (351).

Since the Moloney cell surface antigen (MCSA) is the subject of part of the experimental work described in this thesis, this antigen will be discussed here in more detail. MCSA is found on lymphomas induced by Mo-MuLV in newborn mice (160,161). The "standard" antiserum is raised by injection of heavily irradiated cells of a Mo-MuLV -induced lymphoma (YAC) of A mouse origin into $(A \times C57BL)F_1$ or $(A \times C57L)F_1$ mice (160). MCSA could also be found on Mo-MuLV or R-MuLV -infected JLS-V9 cells (a cell line of BALB/c bone marrow origin; 352), but not on Mo-MuLV or R-MuLV -infected NIH-Swiss/3T3 or BALB/3T3 cells (fibroblasts of whole embryo origin; 311) (307; E.M. Fenyö, personal communication). It should be noted here, however, that we could detect MCSA on Mo-MuLV -infected NIH-Swiss 3T3 cells with membrane immunofluorescence, though at considerably lower levels than on Mo-MuLV -infected JLS-V9 cells (see chapter 6.). Although gp70 and gag-related proteins were shown to be present on the YAC lymphoma cells (against which the antiserum is raised) (85,86,151), E.M. Fenyö and her colleagues obtained several lines of evidence that MCSA is distinct from the viral structural proteins: 1. Two sublines of the lymphoma line made immunoresistant to anti-MCSA by immuno-selection in vivo and in vitro did not express MCSA anymore, but were as sensitive as the parental YAC line to the cytotoxic effect of monospecific heteroantisera against viral structural proteins (85). 2. The cytotoxic effect of anti-MCSA to the YAC cells could not be inhibited by virus or viral proteins (86). 3. In membrane immunofluorescence MCSA did not co-cap with gp70, p15E or H-2. 4. By chromatographic (283) as well as by electrophoretic (314) means MCSA-activity (measured by cytotoxicity-inhibition) could clearly be separated from viral structural proteins and H-2 antigens. On the other hand, MCSA did co-cap with p30 and p12 on the

cell surface in membrane immunofluorescence and could be bound for maximally 15 % to an anti-p15 immunosorbens column (151). Moreover, on a lectin affinity column part of p15 co-chromatographed with MCSA (283). This led to the speculation (151), that MCSA would closely resemble FOCMA and that it is synthesized as a polyprotein of p15 (and p12 ?) covalently linked to a non-structural protein (MCSA). This polyprotein would then be further processed to the mature, non-gag-linked MCSA-molecule, that would become loosely associated with the gag proteins on the cell surface (151).
In this thesis (chapter 6), however, we show ample evidence that MCSA is env-related, but distinct from the exogenous Mo-MuLV env proteins. We conclude, that anti-MCSA recognizes some other MuLV env protein. The expression of that protein could be caused by a contaminating defective virus present as a pseudotype in some Mo-MuLV isolates.

The significance of virus-related cell surface antigens in immunosurveillance against tumor development is not yet clear. In mice natural anti-viral antibodies are predominantly directed against gp70 (110,136,137,295). In addition, anti-p30 antibodies can be found at low levels (140). Although virus-induced tumor cells can elicit strong anti-viral antibodies (213,242), it is not clear whether natural antibody production is induced by virus-related CSAs or by viral structural proteins present in the serum. In virus-producing mice viral structural proteins are found in the serum as constituents of the released virions. It was shown that the glycosylated gag polyproteins are not incorporated into virions, but still are shed into the medium in cell culture (66, 175), so it is presumed that these polyproteins can be found in the serum of infected mice too. In fact, gp70- and p30-related antigens are found in the sera of most mouse strains, either virus-producing or not (124,178). Quantitatively, large differences may exist between various mouse strains. The serum of

normal mice contains a xenotropic virus-neutralizing activity, that is not, however, immunoglobulin-mediated (87,181).
The anti-envelope antibodies neutralize the infectivity of the virion (139) and are able to prevent, albeit incompletely, viral replication in vivo and in vitro (59,95,131,132,153,227). Moreover, these antibodies are cytotoxic for virus-induced tumor cells (105, 110,135,138,208).
Not only a humoral response can be elicited by viral envelope proteins, but a cell-mediated cytotoxic activity is also induced (102,138,154,171).
MCSA is related to the tumor-associated transplantation antigen (TATA) of Mo-MuLV -induced lymphomas, since the amount of MCSA present on a particular tumor correlates quite well with the immunosensitivity of the tumor in vivo, as judged by the rejection of small tumor grafts in preimmunized mice (161). In addition it was shown in genetic experiments that mice which could produce high titers of anti-MCSA were better protected against lymphoma transplants (53). There was no correlation between successful tumor rejection and anti-viral response.
A system in which it was incontrovertibly settled that a virus-related CSA is the main target in natural tumor rejection, is the FeLV/FeSV infected cat system. FeLV/FeSV is endemic in cats (312). Titers of antibodies to FOCMA correlate inversely with the rate of tumor progression (70). On the other hand, the level of virus-neutralizing antibodies does not appear to influence the course of the disease to a great extent (70), although passive immunization with anti-FeLV can temporarily prevent the development of FeLV/FeSV -induced tumors (59).
So in the only case where an important role of a virus-related CSA in tumor rejection is clear, gp70 is not the target, although this antigen and antibodies to it are most widespread. However, virus-induced tumors might not only grow by cell proliferation, but also by virus spread and transformation of new cells (232). Therefore, a humoral and cellular response against the virus could be effica-

cious in preventing the development of tumors.

It should be kept in mind, that leukemia viruses are only leukemogenic when injected into newborn animals, suggesting that adult animals can build up an efficient immunological defense against these viruses and/or neoplasias they induce (108,312). When young mice are infected with leukemia virus they develop tumors, after a latent period. Concomitantly, their immune response becomes generally impaired (38), apparently because the immunoreactive cells are the targets for the leukemogenic activity of the infecting virus (108,312). Moreover, the impairment of the immune system may also be related to the finding mentioned before, that anti-viral antibodies are able to suppress the immune response (197).
In the latent period before tumor outgrowth, tumor development could be blocked by circulating antibodies and cellular immunity. This immunological barrier would then be overcome by the rapid appearance of a highly oncogenic new virus type: the MCF recombinant virus. A model for the mechanism by which this type of virus could induce tumor formation (namely by mitogenic stimulation of specific MCF-receptor bearing target cells) has been described already in section 2.5. Perhaps this virus, or the CSAs present on the cells that express the virus, elicit antibodies that enhance tumor growth, either by blocking cell-mediated cytotoxicity or by suppressing general immunoreactivity of lymphoid cells through interaction with specific viral envelope proteins on these cells.
Since this is still all hypothetical, it is clear, that further studies (e.g. on the quantity and quality of virus and tumor cell antibodies in the serum during the lifespan of AKR mice or neonatally infected mice) are needed.

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3 IDENTIFICATION OF RAUSCHER MURINE LEUKEMIA VIRUS-SPECIFIC mRNAs FOR THE SYNTHESIS OF GAG- AND ENV-GENE PRODUCTS

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Identification of Rauscher murine leukemia virus-specific mRNAs for the synthesis of *gag*- and *env*-gene products

(protein synthesis/oocytes/radioimmunoprecipitation/gel electrophoresis)

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ABSTRACT **Polyadenylated mRNA isolated from cells infected with Rauscher murine leukemia virus was fractionated by centrifugation in a denaturing sucrose gradient into different sizes Each RNA fraction was injected into oocytes of *Xenopus laevis* and the virus-specific products were analyzed by immunoprecipitation with polyvalent and monospecific antisera against polypeptides of Rauscher murine leukemia virus, and then by gel electrophoresis and scintillation autoradiography It was shown that a 35S mRNA species directs the synthesis of a precursor of the internal or group specific antigens of the virion (the *gag*-gene products) A 22S mRNA species directs the synthesis of two viral envelope polypeptides and their precursor polypeptide (*env*-gene products) The results indicate that the *gag* and *env*-related polypeptides of Rauscher murine leukemia virus are synthesized uncoordinately and provide evidence for open and closed cistrons on the virus-specific mRNAs**

The structural polypeptides of the murine leukemia viruses and presumably also those of all other RNA tumor viruses are subgene products derived from the primary translation products precursor polypeptides of the *gag* and the *env* gene (for a review see ref 1) In our studies with JLS V9 cells infected with Rauscher murine leukemia virus (R MuLV) we have identified a glycosylated polypeptide with a molecular weight of 82 000 (*env* pr82) as the precursor of two polypeptides of the viral envelope gp69 71 and p15(E) (2 3) The polypeptide p12(E) (designated p12 in ref 3) shares chymotryptic peptides with p15(E) therefore p12(E) is also derived from the *env* precursor polypeptide (our unpublished results and ref 4) In the same studies (2 3) we recognized two mutually related polypeptides with molecular weights of 75 000 (called *gag*-pr75) and 65 000 (called *gag* pr65) as the precursors of the internal virion polypeptides p30 and p15 Recently Barbacid *et al* (5) demonstrated that the two other internal polypeptides p12 and p10 are also derived from the precursor of p30 and p15 All these data are consistent with those reported by several other authors (4 6–9)

The existence of virus specific mRNA species of different size classes (10) suggests that the synthesis of *gag* and *env* precursor polypeptides is programmed by different mRNAs rather than by one polycistronic mRNA In agreement with this suggestion it was shown that each virus specific mRNA species mediated the synthesis of its own virus specific product(s) in a rabbit reticulocyte cell free system (11) Of the two virus-specific products formed in this system under the direction of the 35S mRNA fraction one comigrated during sodium dodecyl sulfate (NaDodSO$_4$) polyacrylamide gel electrophoresis with the *gag* pr65 mentioned above the other one with *gag* pr72 a *gag* related polypeptide found in infected cells in the presence of the arginine analog canavanine (3) Similar products were found in various protein synthesizing systems programmed with RNA isolated from virions of RNA tumor viruses and identified as *gag* related products (12 17) In none of these experiments with virion RNA however, could *env* related products be detected

In addition to these results obtained with genome size virus specific RNA (35S) (whether isolated from virions or as polyadenylylated mRNA from infected cells) two observations described in our previous study are noteworthy (a) the 22S mRNA fraction isolated from cells infected with R MuLV mediated the synthesis of a virus specific 70 000-dalton polypeptide in a cell free system from reticulocytes (11) a polypeptide of the same size as the *env* related product formed in infected cells when glycosylation is inhibited (ref 9 and W J M van de Ven unpublished) (b) in an endogenous cell free system derived from cells infected with R MuLV free polyribosomes containing 35S RNA as the main virus-specific mRNA produced *gag*-products only whereas the membrane bound polyribosomes gave rise to both *gag* and *env* products and contained 22S virus specific mRNA in addition to the 35S RNA (11) These results prompted us to localize the *gag* and *env*-messenger activity among the heterologous virus-specific mRNAs

MATERIALS AND METHODS

Cells and Virus The JLS-V9 cell line derived from bone marrow cells of Balb c mice infected with and producing R MuLV was grown in Eagle's basal minimal essential medium supplemented with 10% calf serum (18) Labeling of JLS V9 cells and preparation of cell lysates for immunoprecipitation was described elsewhere (3)

Isolation of Polyribosomes. For the preparation of total polyribosomes cells were treated and subsequently harvested as described before (11) After the cells (2–3 ml) were washed and swollen in hypotonic buffer (11) they were disrupted by 10 strokes of a Dounce homogenizer (B pestle) in 12 ml of lysis buffer (50 mM Tris HCl pH 7 4 $Mg(Ac)_2$ 5 mM KAc 150 mM Nonidet P-40 1% (vol vol) sodium deoxycholate 0 5% (wt vol) dithiothreitol 2 mM and 100 μg ml of an RNase inhibitor from bovine eye-lens purified on DEAE cellulose (obtained from H Bloemendal) Nuclei and cell debris were removed by centrifugation for 4 min at top speed (20 000 rpm) in a Ti50 rotor (Beckmann) The supernatant was layered onto 1 0 ml of 2 M sucrose in 20 mM Tris-HCl pH 7 4 KAc 120 mM $Mg(Ac)_2$ 5 mM and 2 mM dithiothreitol and polyribosomes were collected by centrifugation for 2 5 hr at 56 000 rpm in an SW 56 rotor (Beckmann)

Isolation and Fractionation of mRNA. Polyadenylylated mRNA was isolated from total polyribosomes by affinity chromatography on oligo(dT)-cellulose as described (11) with

Abbreviations R MuLV Rauscher murine leukemia virus NaDodSO$_4$ sodium dodecyl sulfate *gag* and *env* are each viral genes

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the following modifications polyribosomes were incubated in buffer A for 10 min at 37° with Pronase(RNase free) 0.5 mg/ml and NaDodSO$_4$ was omitted from column buffer B. mRNA was fractionated by centrifugation in a denaturing sucrose gradient into various sizes as described in the legend to Fig. 1.

Antisera. Antisera raised against R-MuLV polypeptides and used for immunoprecipitation were described elsewhere (3). We observed no cross-reactivity among the monospecific antisera used in this study; a slight coprecipitation however of gp69/71 p15(F) and p12(F) was observed in immunoprecipitation reactions with antiserum against gp69/71 and antiserum against both p15(E) and p12(F) (designated anti-p15(F)-p12 serum in ref. 3) due to an association of these viral envelope polypeptides (3). The antiserum against p15(E)-p12(F) was raised against a mixture of p15(F) and p12(I) (3). Moreover it is now known that p15(F) and p12(F) contain common amino acid sequences (D. Van Zaane, unpublished results and ref. 4). To rule out contamination with antibodies against reverse transcriptase we tested the antisera against R-MuLV gp69/71 and p30 for their capacity to inhibit the enzyme activity. The sera did not react in this test (not shown) and therefore they will probably not immunoprecipitate reverse transcriptase.

RESULTS

Virus-Specific mRNA Activity of Total Polyadenylylated RNA from Infected Cells. Translation of 35S RNA isolated from R-MuLV in oocytes of *Xenopus laevis* has clearly demonstrated that this RNA preparation directs the synthesis of *gag* pr65 only (15). In a preliminary experiment with these oocytes we observed that a mixture of virus-specific mRNAs of different size classes (14 S, 22 S and 35 S) isolated from JLS-V9 cells infected with R-MuLV mediated the synthesis of the *gag* gene product *gag* pr65, as well as the *env* gene products *env* pr82, gp69/71 and p15(E) (not shown). These observations indicate that at least two separate mRNA functions are present among the population of virus-specific mRNAs, a *gag*-specific and an *env*-specific mRNA. To identify both mRNA activities we repeated this experiment with fractionated mRNA.

Virus-Specific mRNA Activity of Fractionated mRNA. The fractionation of mRNA from infected cells was performed by sucrose gradient centrifugation in the presence of formamide as a denaturing agent. The sedimentation pattern of virus-specific mRNA in the gradient was determined by hybridization with DNA complementary to R-MuLV RNA (Fig. 1). The results are essentially the same as those reported before (11) and the virus-specific mRNA species of 14 S, 22 S and 35 S can be distinguished. Oocytes of *Xenopus laevis* were injected with RNA from each fraction and incubated with [^{35}S]methionine. The newly synthesized virus-specific polypeptides were separated from the large excess of JLS-V9-specific polypeptides and endogenous oocyte polypeptides by immunoprecipitation with anti-R-MuLV serum; this serum reacts with both *gag* and *env* related products (3). The immunoprecipitates were analyzed by NaDodSO$_4$-polyacrylamide gel electrophoresis and scintillation autoradiography (Fig. 2). Three polypeptides, two in the 50 000 dalton region and one with a very high molecular weight, were precipitated in a control experiment with oocytes injected with water (Fig. 2 *slot 5*) as well as in all other analyses of injected oocytes (Fig. 2 *slots 6–19*). Apparently these polypeptides are not virus-specific and therefore will be ignored in further discussions. A number of virus-specific polypeptides can be recognized which comigrate with the R-MuLV-specific precursor polypeptides or with the mature virion polypeptides

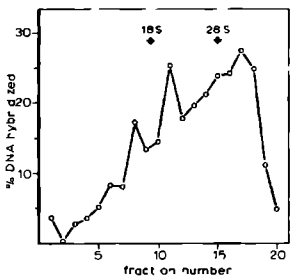

FIG. 1. Sucrose gradient centrifugation of virus-specific mRNAs from JLS-V9 cells infected with R-MuLV. Polyadenylylated mRNA (10 μg) isolated as described in *Materials and Methods* section was denatured at 56° for 3 min with formamide (85% (vol/vol) in 2 mM Tris-HCl pH 7.4/1 mM EDTA) 1 mM/and 0.5% NaDodSO$_4$ (wt/vol). After dilution with one volume of TNE (10 mM Tris-HCl pH 7.4/NaCl 100 mM/EDTA 1 mM) the RNA preparation was layered onto an isokinetic sucrose gradient in TNE containing 50% (vol/vol) formamide [C (top) = 5% (wt/vol) C$_b$ (high) = 14.4% (wt/vol) V$_n$ (ml) = 12.9 ml]. The tubes were spun in a SB 283 rotor (IEC) for 13 hr at 4° at 41 000 rpm. RNA from each fraction was precipitated with alcohol after addition of 10 μg of 28S RNA from calf eye lens ribosomes as a carrier. The relative amount of virus-specific RNA was determined in each fraction by hybridization with DNA complementary to R-MuLV RNA (10).

immunoprecipitated from infected cells (Fig. 2 *slots 20* and *21*).

The autoradiogram of virus-specific translation products (Fig. 2) suggests a sharp separation of virus-specific mRNA which is in contrast with the diffuse hybridization pattern depicted in Fig. 1. This may be explained by the nonlinear blackening of the film during scintillation autoradiography and by the unknown representation of the viral RNA in the cDNA probe used for hybridization.

Identification of the *gag* mRNA. In agreement with our previous study in which we used a cell-free system from reticulocytes (11), the 35S mRNA species (Fig. 1, fraction nos. 17 and 18) directed the synthesis of a 65 000 dalton polypeptide (Fig. 2 *slots 17* and *18*). Fig. 3*I*,*K* shows that the 65 000 dalton polypeptide synthesized in oocytes can be precipitated with antiserum against p30 as well as with antiserum against p15 but not with antiserum to gp69/71. Incidentally it should be noted here that the antiserum against gp69/71 precipitates a polypeptide (about 32 000 daltons) from oocytes injected either with the various RNA fractions (Fig. 3*F*,*F* and *K*) or with water (Fig. 3*I*), indicating that this polypeptide is an endogenous oocyte product. We conclude that the 65 000-dalton polypeptide is similar if not identical to the *gag* gene product *gag* pr65 found in infected cells. This conclusion is consistent with a reverse experiment carried out by Muller-Lantzsch (21) showing that the polyribosomes taken from cells infected with Moloney murine leukemia virus that were immunoprecipitated with antiserum against p30 contained 35S RNA as the main virus-specific RNA species.

Identification of the *env* mRNA. The 22S mRNA fraction (Fig. 1, fraction no. 11) mediates the synthesis of three polypeptides (Fig. 2 *slot 11*) which were identified as the *env* gene products *env* pr82, gp69/71 and p15(E) they comigrated with the *env* gene products found in infected cells (Fig. 2 *slots 20* and *21*). The newly synthesized polypeptide comigrating with *env* pr82 could be immunoprecipitated with antiserum against gp69/71 and antiserum against p15(E)-p12(F), whereas those

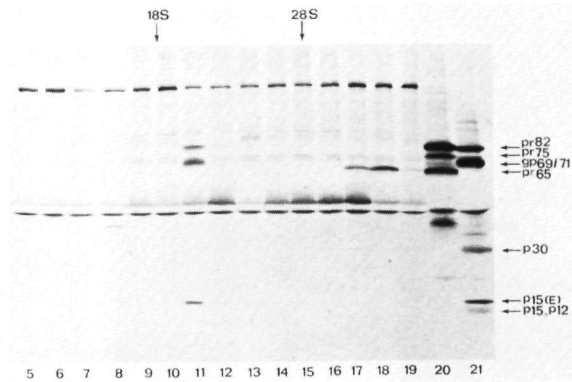

FIG. 2. Radioimmunoprecipitation analysis with antiserum against R-MuLV of the virus-specific products synthesized after injection of oocytes of *Xenopus laevis* with fractionated polyadenylylated RNA from JLS-V9 cells infected with R-MuLV. RNA precipitated from each fraction of the gradient shown in Fig. 1 was dissolved in 10 μl of H_2O; 0.5 μl was injected into 20 oocytes (19). The oocytes were incubated for 20 hr at 20° with 25 μCi of L-[^{35}S]methionine (specific activity, 329 Ci/mmol; from The Radiochemical Centre, Amersham, England) and, subsequently, homogenized in 1.25 ml of immunoprecipitation buffer (3). The homogenate was cleared by centrifugation for 10 min at 220,000 × *g* in a Ti50 rotor (Beckman). Virus-specific polypeptides from the supernatant were analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis (11) and scintillation autoradiography (20) after indirect immunoprecipitation with antiserum against R-MuLV (2, 3). *Slots 6–19* correspond with the fraction numbers of the gradient shown in Fig. 1. *Slot 5*: (control) (immunoprecipitation analysis with antiserum against R-MuLV of products synthesized after injection of 0.5 μl of H_2O into 20 oocytes). *Slot 20*: virus-specific polypeptides immunoprecipitated with antiserum against R-MuLV from infected JLS-V9 cells treated for 15 min with a ^{14}C-labeled amino acid mixture (3). *Slot 21*: the same as slot 20 except that the radioactivity was chased for 8 hr with an excess of unlabeled amino acids (3).

comigrating with gp69/71 and p15(E) were immunoprecipitated with the corresponding antisera (Fig. 3*F* and *G*). Fig. 3*H* shows that the *gag*-specific antiserum against p30 does not precipitate translation products of 22S mRNA.

Immunoprecipitable Translation Products of the 14S and 25S mRNA Fractions. The 45,000-dalton polypeptide formed after injection of 14S RNA (Fig. 1, fraction no. 8) and immunoprecipitated with the antiserum against R-MuLV (Fig. 2, *slot 8*) was not precipitated with the monospecific antiserum against p30, antiserum against p15, or antiserum against gp69/71 (Fig 3*C–E*). The nature of this product remains unknown; we cannot exclude the possibility that it is coded for by a host 14S mRNA. Finally, the 25S mRNA fraction (Fig. 1, fraction no. 13) directs the synthesis of a minor polypeptide (85,000–90,000 daltons) which was precipitated with antiserum against R-MuLV (Fig. 2, *slot 13*) but not with antiserum against p30 or antiserum against gp69/71 (not shown). This polypeptide could represent a host-specific product from JLS-V9 cells recognized by the antiserum against R-MuLV. Some unidentified polypeptides in the same size range were precipitated by the antiserum against R-MuLV when 25–28S mRNA from infected cells was translated in the reticulocyte system (11).

DISCUSSION

Open and Closed Cistrons on Oncornaviral mRNA. In 1974, Baltimore (22) proposed four "genetic elements" for the avian sarcoma viruses: *gag* (for the precursor polypeptide of the major core protein), *env* (for the major envelope protein), *pol* (for the RNA dependent DNA polymerase) and *onc* (for cell transformation). He discussed them "as if they were genes constituting most of the genome of an RNA tumor virus." Since that time, all known structural polypeptides of the murine leukemia viruses could be assigned to either the *gag*- or the *env*-gene. These two genes, therefore, contain "subgenes"; their primary translation products are precursor polypeptides that are cleaved into subgene products.

Our present results show that each of the two genes, *gag* and *env*, is expressed by translation of its own specific mRNA. Such virus-specific mRNAs although functionally monocistronic may apparently overlap other genes and, therefore, be structurally polycistronic. This is obvious for the genome size 35S *gag*-mRNA and probable for the 22S *env*-mRNA with its estimated molecular weight of 1.2×10^6 (10). Similar conclusions follow from hybridization data on oncornaviral mRNAs of different size classes (10, 23–25). It is tempting to speculate that as a rule the open (i.e., translated) cistron would be the 5′-terminal one. Otherwise a more complicated model would be required to explain the recognition of the open initiation site. For the *gag*-mRNA, this speculation would be in agreement with the probable gene order proposed for the avian RNA tumor viruses, 5′-*gag-pol-env-onc*-poly(A)-3′ (26). By assuming the same gene order the *env*-mRNA would then have the structure 5′-*env-onc*-poly(A)-3′.

Much less is known on the expression of *pol* and *onc*; the latter gene although identified from mutants of murine sarcoma viruses (27) is still hypothetical in the case of leukemia viruses. If *pol* and *onc* also have separate mRNAs the model predicts the following structures: 5′-*pol-env-onc*-poly(A)-3′ and 5′-*onc*-poly(A)-3′.

Very Large Virus-Specific Polypeptides. The concept of separate mRNAs for the *gag*- and the *env*-gene agrees with data on the uncoordinate expression of p30 and gp69/71 in cells harboring endogenous or defective RNA tumor viruses (28, 29). Likewise, some recent experiments in which the initiation of protein synthesis was influenced by high salt conditions (30) indicated that different sites for the initiation of protein synthesis are operative in the expression of the *gag*- and the *env*-gene of nondefective mouse mammary tumor virus. From these experiments and from the results described in this paper, we

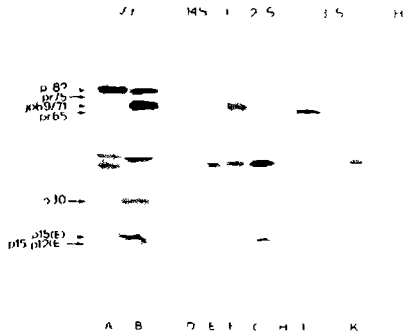

FIG. 1 Monospecific antisera identification of R MuLV specific products formed in oocytes of *Xenopus laevis* after injection with mRNA fractions from HSV9 cells infected with R MuLV Technical details and fraction numbers of mRNA are as described in the legend to Fig. 2 Lysates of oocytes injected with 14S mRNA (fraction no 8 *slots C F*) 22S mRNA (fraction no 11 *slots E H*) and 35S mRNA (fraction no 18 *slots I K*) were analyzed with monospecific antisera The following monospecific antisera were used antiserum against p30 (*slots C H I* and *I*) antiserum against p15 (*slots D J* and *M*) antiserum against gp69/71 (*slot E F K* and *N*) antiserum against p15(E) p12(E) (*slot G*) As a control similar analyses were performed with oocytes injected with H O (*slots I A*) and is a reference the virus specific polypeptides from infected cells are shown (*slots A* and *B*) (*Slot A*) Virus specific polypeptides immunoprecipitated with antiserum against R MuLV from lysates of HSV9 cells infected with R MuLV treated for 15 min with ^{14}C labeled amino acids (*Slot B*) The same as *slot A* except that the radioactivity was chased for 8 hr with an excess of unlabeled amino acids

conclude that translation of the genome of RNA tumor viruses into one giant precursor polypeptide (250 000–300 000 daltons) as found for picornaviruses (31 32) is unlikely or at least is not the principal pathway for the synthesis of the virus specific polypeptides Some authors have noticed varying amounts of large virus specific polypeptides (>100 000 daltons) in infected cells (8 9) their physiological significance however remains obscure

Synthesis of the *env* and *gag* Products in the Oocyte System In our previous study a 70 000-dalton polypeptide was synthesized in a cell free system from reticulocytes after the addition of 22S mRNA isolated from cells infected with R MuLV (11) Presumably this polypeptide was the protein moiety of the glycosylated *env* pr82 (3) because a 70 000-dalton polypeptide was also found in infected cells when glycosylation was inhibited with D 2 deoxyglucose or cytochalasin B (ref 9 W J M van de Ven unpublished results) In oocytes of *Xenopus laevis* however the *env* products were apparently glycosylated because they comigrated with *env* pr82 and gp69 71 The synthesis of *env* pr82 was also observed in an endogenous cell free system from infected cells containing polyribosomes still attached to membranes 11) It is generally accepted (33) that an enzyme system associated with the endoplasmatic reticulum recognizes signals for the glycosylation of nascent polypeptides This enzyme system is apparently well conserved during the evolution of the vertebrates The same seems to be true for another posttranslational activity the cleavage of precursor polypeptides because in addition to *env* pr82 the subgene products gp69 71 and p15(E) are also formed in the amphibian oocytes The *gag* related polypeptides were less well represented in oocytes although in some experiments a partial processing of *gag* pr65 was observed (not shown) In the experiments presented here *gag* pr65 was the sole *gag* specific product formed in the oocytes in infected cells we had previously identified a polypeptide called *gag* pr75 is the immediate precursor to *gag* pr65 (3)

It should be noted that the independent confirmation of our conclusions by tryptic peptide analysis has not been undertaken because the low amount of radioactivity that was incorporated in virus specific products (approx 500 cpm 20 oocytes)

Translation of 14S RNA in the Oocyte System In contrast to the 45 000 dalton polypeptide synthesized after injection of 14S mRNA from infected cells into oocytes one 30 000 and two 15 000 dalton polypeptides were produced in a cell free system from reticulocytes in response to the same mRNA fraction (11) Although it is remarkable that all these products (precipitable with antiserum against R MuLV are formed under the direction of an mRNA fraction that coincides with a small peak in the hybridization profile obtained with R MuLV cDNA (Fig 1) the origin the nature and the possible relationship between these polypeptides are not yet clear

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4. IDENTIFICATION OF VIRUS-SPECIFIC mRNA SYNTHESIZING MURINE LEUKEMIA VIRUS RNA-DEPENDENT DNA POLYMERASE (REVERSE TRANSCRIPTASE)

IDENTIFICATION OF VIRUS-SPECIFIC mRNA SYNTHESIZING MURINE LEUKEMIA VIRUS RNA-DEPENDENT DNA POLYMERASE (REVERSE TRANSCRIPTASE)

ABSTRACT

In order to investigate the size-class of virus-specific messenger RNAs that are involved in the synthesis of the murine leukemia virus (MuLV) enzyme reverse transcriptase (RT), we translated size-fractionated mRNAs from Moloney-MuLV -infected cells in oocytes of Xenopus laevis. Reverse transcriptase was either detected by its enzymatic activity or by immunoprecipitation. Although we were not able to detect reverse transcriptase activity in oocytes injected with mRNAs from virus-infected cells, we did precipitate reverse transcriptase molecules from lysates of injected oocytes. It appeared that reverse transcriptase was synthesized on genome-size mRNA. These results are in accordance with those obtained earlier by translating virion RNA and with recent results of translation studies with cellular mRNAs in cell-free systems. This chapter, however, shows for the first time the synthesis of mature reverse transcriptase in vitro.

INTRODUCTION

The genome of mammalian non-defective RNA tumor viruses contains three known genes: gag, encoding the structural proteins of the viral core, pol, encoding the viral reverse transcriptase, and env, encoding the proteins of the viral envelope (4).
The genome order has never been formally proven for mammalian viruses, but most probably it is the same as for the avian leukosis virus genome: 5'-gag-pol-env-poly(A)-3' (17,48,49,50). It has been shown that the gag gene is translated from a virus-specific messenger RNA with the same size and genetic content as the 35S genomic RNA (15,26,32,37,38,46; chapter 3.). The viral envelope proteins are synthesized on a 22S size mRNA (37,38,46; chapter 3.). The 22S virus-specific mRNA arises by splicing of the genomic RNA: it contains both the ultimate 5' end of the 35S RNA (at least 150 nucleotides long; 35) and the 3' half of the genome (7,11,19,24, 34,35,51).

The primary gene product of the pol gene is not the mature reverse transcriptase molecule itself, but a precursor polyprotein encompassing both gag and pol sequences with a molecular weight of 180,000-200,000, $Pr180\text{-}200^{gag\text{-}pol}$ (16,18,53). In the first processing steps the gag sequences are split off and through several intermediates the mature polymerase (molecular weight 70,000-80,000) is formed (16,18). The presence of gag sequences in the precursor to reverse transcriptase requires that the mRNA coding for this polyprotein would at least contain both gag and pol sequences. This is contradictory to the structure we tentatively proposed earlier for the pol-mRNA (46; chapter 3.): 5'-pol-env-(onc)-poly(A)-3'. Translation of virion RNA *in vitro* showed, that the gag-pol precursor polyprotein could be synthesized by genome-size RNA (23,27,30,33,52). Philipson et al. (33) were able to augment the production of the murine leukemia virus (MuLV) gag-

pol precursor by the addition of yeast suppressor tRNA to the cell-free system. Therefore, they proposed that the precursor to reverse transcriptase would be made on the same RNA molecule as the gag (precursor) polyproteins $Pr65^{gag}$ and $gP80^{gag}$ are made on, by a process of incidental "read-through" of the gag termination codon. Weiss et al. (52), however, did not detect any effect of yeast suppressor tRNAs on the synthesis of the gag-pol precursor of avian sarcoma virus (ASV). They suggested a separate pol-mRNA identical to the gag-mRNA, except for a small sequence that would be spliced out from one of the mRNAs. Thus, a termination codon would be present at the end of the gag gene in the gag-mRNA and absent in the pol-mRNA. A final answer on this point is not yet available. Suppression of a termination codon present on mammalian cell mRNA by yeast suppressor tRNAs in a heterologous cell-free system seems to be rather artificial, yet suppressor tRNAs have also been found in normal mammalian cells (12) and may have a significant function in the fine regulation of gene expression in higher eukaryotes. In order to investigate whether reverse transcriptase was also made on genome-size mRNA in the infected cell, we translated size-fractionated mRNA from Moloney murine leukemia virus (Mo-MuLV) -infected cells in living oocytes of the African clawed frog Xenopus laevis. Detection of reverse transcriptase was attempted by two means: enzymatically, by measuring the polymerase activity in a poly(A).oligo(dT) template.primer system, or by immunoprecipitation with an antiserum directed against reverse transcriptase, followed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

MATERIALS AND METHODS

Materials. L-(^{35}S)methionine (spec. act. 800-1200 Ci/mmol), (U-^{14}C)protein hydrolysate (spec. act. 54 mCi/matom carbon) and (methyl-^{3}H)thymidine 5'-triphosphate (40-60 Ci/mmol) were obtained from Radiochemical Centre, Amersham, England. Oligo(dT)-

cellulose (T-2 and T-3), poly(A) and oligo(dT)$_{12-18}$ were from Collaborative Research Inc., Waltham, Mass.. Heparin (sodium salt, medical grade; 160 U/mg) was purchased from Organon, Oss, The Netherlands. Proteinase K was from Boehringer, Mannheim, West-Germany. Protein A-Sepharose CL-4B was from Pharmacia, Uppsala, Sweden.

Antisera. The rabbit antiserum directed against Mo-MuLV reverse transcriptase (anti-RT) was a generous gift of Drs O.N. Witte and D. Baltimore. According to their findings this antiserum contained contaminating anti-p15 activity. Rabbit antisera against disrupted R-MuLV and (R-MuLV)p15E,p12E were prepared and described by Van Zaane et al. (45).

Cells and virus. In this study the clonal NIH-3T3 line NCL5611P3 (a gift of Dr J.R. Stephenson) infected with Mo-MuLV was used. Cells were grown in monolayer cultures in Dulbecco's modified Eagle's medium with Earle's salts supplemented with 10 % newborn calf serum. Mo-MuLV was isolated from concentrated medium of clone 1A cells (a clonal Mo-MuLV -infected NIH-3T3 line; 10) by the method of Duesberg and Robinson (9).

Cell labeling and lysis procedure. Cells were labeled as described earlier (44,45). In short, cells were grown to about 80 % confluency, starved for 10 min in labelmedium (see below) without radioactive amino acids and labeled in 0.08 ml/cm^2 Hanks' balanced salt solution supplemented with 10 % dialyzed calf serum, vitamins, essential amino acids (except for the radioactive ones) and 50 μCi/ml L-(^{35}S)methionine or 25 μCi/ml (U-^{14}C)protein hydrolysate (labelmedium). After 15 min the labelmedium was removed and replaced by normal medium. Now the cells were either lysed or radioactivity was chased for 8 h. Lysis was performed by the addition to cells and medium of 1/4 volume of fivefold concentrated immunoprecipitation buffer (see below). The lysate was forced 10 times through a 21 gauge needle and cleared by centrifugation at 220,000 x g for 15 min. Lysates were stored at -20 ^{o}C when used within three months or at -80 ^{o}C for longer periods.

Isolation and fractionation of mRNA. All solutions were treated with 0.1 % diethyl pyrocarbonate and (except for the sucrose solutions) autoclaved; glassware was heat-sterilized. Indicated pH values are at 25 °C.

NIH-3T3(NCL5611P3)/Mo-MuLV cells were grown to 80 - 90 % confluency, removed from the culture flasks by trypsinization and washed twice with isotonic buffer (10 mM Tris-pH 7.2, 146 mM NaCl). RNA was isolated from these cells by the magnesium-precipitation method of Palmiter (29) at 0 - 2 °C. Cells were quickly swollen in hypotonic buffer (25 mM Tris-pH 6.9, 25 mM NaCl, 5 mM $MgCl_2$) and then lysed by forcing them through a 21 gauge needle in 9 ml lysis buffer (hypotonic buffer containing 2 % Triton X-100 and 1 mg/ml heparin) per gram of unswollen cells. After 10 min on ice the lysate was centrifuged for 10 min at 12,000 x g. To the supernatant one volume of precipitation buffer (four volumes of lysis buffer plus one volume of 1 M $MgCl_2$) was added and the RNA was let to precipitate for one hour on ice. The precipitate was collected by centrifugation in 30 ml siliconized glass tubes through a layer of 5 ml of 0.2 M sucrose in hypotonic buffer for 30 min at 20,000 x g. The pelleted RNA was dissolved in 10 mM Tris-pH 6.9, 20 mM NaCl, 10 mM EDTA and 0.5 % SDS to a concentration of 1 - 2 mg/ml and treated with preincubated (1 h at 37 °C, 1 mg/ml in 10 mM Tris-pH 6.9, 20 mM NaCl) proteinase K (100 µg/ml) for 15 min at 37 °C. The solution was then heated for 2 min at 100 °C, chilled, brought to 0.5 M NaCl final concentration, and applied on an oligo(dT)-cellulose (T-2) column (3). The column was washed with 10 mM Tris-pH 7.4, 0.5 M NaCl, 1 mM EDTA, 0.5 % SDS until A_{260} was below 0.02, then washed with 10 mM Tris-pH 7.4, 0.25 M NaCl, 1mM EDTA, and the poly(A)-tagged RNA was eluted with 10 mM Tris-pH 7.4. This procedure was performed at 25 °C. The mRNA was precipitated overnight at -20 °C in siliconized glass tubes by the addition of 1/10 volume of 2 M NaAc-pH 5.2 and 2 volumes of ethanol. The precipitate was collected by centrifugation for 30 min at 10,000 x g and -20 °C, the pellet was dried in vacuo, and the RNA was dissolved, heated and

cycled on oligo(dT)-cellulose for a second time. The thus selected mRNA was alcohol-precipitated and stored at -20 °C. Messenger RNA from NIH-3T3(NCL5611P3) cells infected with cloned Rauscher-MuLV wild type virus (wt-248) or a mutant of R-MuLV with temperature-sensitive reverse transcriptase (ts-29) (39,42) was isolated in the same manner and given to us by Dr W.J.M. van de Ven from our laboratory. The viruses wt-248 and ts-29 were originally obtained from Dr J.R. Stephenson.

The mRNA (50 - 150 /ug) was fractionated according to size by centrifugation for 6.5 h at 40,000 rpm and 20 °C in an IEC SB-283 rotor on a 15 - 37.5 % (w/w) isokinetic sucrose gradient (43) in low salt buffer (20 mM NaAc-pH 5.2, 2mM EDTA, 0.2 % SDS). Prior to centrifugation the RNA was denatured in low salt buffer containing 85 % formamide by incubation for 5 min at 37 °C and 30 sec at 68 °C. Fractions (0.5 ml) from the gradient were collected and alcohol-precipitated after the addition of 10 /ug rat lens 28S ribosomal RNA as a carrier.

Translation of mRNA in Xenopus oocytes. Messenger RNA was translated in Xenopus laevis oocytes as described by Asselbergs et al. (1). Unfractionated mRNA was dissolved to 1 mg/ml, mRNAs from gradient fractions were dissolved in 10 - 20 /ul distilled water and 40 nl of the solution was injected per oocyte. When RT had to be detected by its enzymatic activity oocytes were incubated for 16, 40 or 64 h at 19 °C in modified Barth's medium. Lysis of these oocytes is described below. For immunoprecipitation studies 20 oocytes per fraction were injected and labeled for 20 h at 19 °C in 100 /ul modified Barth's medium containing 1 mCi/ml L-(^{35}S)-methionine. These oocytes were lysed in 1 ml of immunoprecipitation buffer (see below), using a small Potter-Elvehjem -type homogenizer. The lysates were cleared by centrifugation for 15 min at 220,000 x g and carefully aspirating the lipid containing upper layer, and they were stored at -20 °C.

Enzymatic detection of reverse transcriptase in oocyte lysates. Reverse transcriptase activity could not be measured directly in

the lysates of oocytes, since under these conditions activity was completely blocked. After a number of pilot experiments we decided to the following procedure that was based on the method developed by Gerwin and Milstien (14). Indicated pH values are at 25 °C. After incubation the injected oocytes were washed with fresh Barth's medium, succed dry, and per 50 oocytes 100 µl of buffer A (50 mM Tris-pH 7.4, 5 mM $MgAc_2$, 0.1 mM EDTA, 0.1 M 2-mercaptoethanol, 0.2 % NP-40 and 25 % (w/v) glycerol; ref. 47) made to 0.5 M KCl, and 10 µl NP-40 and 10 µl of 10 % (w/v) sodium deoxycholate was added. Lysates were prepared by 20 strokes in a small Potter-Elvehjem homogenizer. The lysate was diluted to 1 ml with buffer A (50 mM KCl final concentration) and stored at -20 °C. About 25 mg of dry oligo(dT)-cellulose (T-3) per ml of lysate to apply was swollen in 0.1 M NaOH, washed with 0.1 M NaOH and equilibrated with buffer A. The lysate was added and the reverse transcriptase was let to bind to the oligo(dT)-cellulose, while shaking every 5 min. This and the following steps were carried out at 0 - 2 °C in 1.5 ml conical polypropylene centrifuge tubes. After one hour the tubes were centrifuged for 1 min at 12,000 x g, the supernatant was removed, and the oligo(dT)-cellullose was washed four times for 2 min with 0.5 ml of buffer A, and four times with 0,5 ml of buffer A containing 100 mM KCl. Reverse transcriptase was eluted by washing the oligo (dT)-cellulose eight times for 2 min with 0.2 ml of buffer A containing 600 mM KCl.

From each fraction 20 µl was tested for reverse transcriptase activity. The assay system (70 µl) contained (in addition to the concentrations contributed by the samples): 45 mM Tris-pH 8.2, 0.25 mM $MnCl_2$, 2 mM dithioerythritol, 0.3 A_{260}/ml of poly(A), 0.1 A_{260}/ml of oligo(dT) and 5 µCi (5 µM) of (methyl-^{3}H)thymidine 5'-triphosphate. The final concentration of KCl was corrected to 170 mM when necessary. Incubation was at 37 °C. After one hour 0.5 ml of 0.1 M $Na_4P_2O_7$, 40 µg of yeast RNA, and 0.5 ml of 20 % trichloric acetic acid was added to precipitate the multimeric nucleic acids. Precipitates were collected on nitrocellulose

filters and radioactivity was counted in a liquid scintillation counter. Counting efficiency was about 20 %.

Immunoprecipitation and polyacrylamide gel electrophoresis. Immunoprecipitation of radioactive polypeptides from lysates of mouse cells and Xenopus oocytes was performed as described by Van Zaane et al. (45) in about 0.8 ml of 10 mM sodium phosphate-pH 7.2, 0.9 % NaCl, 1 % Triton X-100, 0.5 % sodium deoxycholate and 0.1 % SDS. About 1-2.5 x 10^5 TCA-precipitable cpm of cellular lysates and about 1-2.5 x 10^6 cpm of oocyte lysates were taken for each precipitation. One /ul of antiserum was added, incubation was for 16 h (overnight) at 0 °C, 50 /ul of a 10 % (v/v) suspension of Protein A-Sepharose CL-4B was added and the mixture was shaken for 30 min at room temperature. The immunoprecipitates were collected by centrifugation through a layer of 0.2 ml of 10 % (w/v) sucrose in immunoprecipitation buffer for 5 min at 12,000 x g and were washed three times with immunoprecipitation buffer. The immunoprecipitates were dissolved by heating them for 5 min at 95 °C in 20 /ul of 62.5 mM Tris-pH 6.8, 2 % SDS. 5% 2-mercaptoethanol, 6 M urea, 10 % glycerol and 0.1 % bromophenol blue, and were applied on the gel. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to Laemmli (20) using 0.75 mm thin 7 - 18 % polyacrylamide gradient slab gels. Radioactivity was visualized after treatment of the gels with dimethylsulphoxide/2,5-diphenyloxazole (DMSO/PPO) (6) by scintillation autoradiography with preflashed (21) Kodak XR films.

RESULTS

Enzymatical detection of reverse transcriptase activity in oocytes injected with mRNA from infected cells.

When we first started these investigations little was known about the biosynthesis of reverse transcriptase. Antisera directed against this viral enzyme were not available to us. We decided to use the

RNA-dependent DNA polymerase activity of reverse transcriptase for its detection. First, we optimized the assay that was routinely used in our laboratory for the detection of retrovirus. In the final form of the assay (see Methods section) an incorporation of 800 pmol (5×10^6 cpm) thymidine 5'-monophosphate with 1 /ug of virus (Mo-MuLV) was obtained. To estimate whether we would have any chance to detect reverse transcriptase activity in this manner in lysates of oocytes injected with mRNA from MuLV-infected cells, we made the calculations as indicated in table 1.

On the basis of this tentative calculation we decided to attempt in this way to determine the size of pol-mRNA. Since poly(A).oligo(dT) is not a template-primer combination that is specific for the viral RNA-dependent DNA-polymerase (although it is the combination that gives the highest TMP incorporation with reverse transcriptase in the exogenous RT assay (25)), we planned to control virus-specificity of detected polymerase activity by using different combinations of synthetic templates and primers (13,36), and by testing the thermosensitivity of the polymerase activity found in oocytes that were injected with mRNA from cells infected with a thermosensitive mutant of R-MuLV, ts-29.

Reverse transcriptase of this mutant has been shown to be more thermolabile than reverse transcriptase of the corresponding wild-type parent (42).

In pilot experiments it appeared that reverse transcriptase activity could not be measured directly in the oocyte lysate. Therefore, we adapted a simple one step purification procedure (14) to our conditions. Reverse transcriptase present in oocyte lysates was bound to oligo(dT)-cellulose, non-bound proteins (and nucleic acids) were removed by extensive washing and the enzyme was eluted with a high salt buffer. A phosphate buffer could not be used for this purpose, since minimal concentrations (1 - 5 mM) of phosphate already inhibited reverse transcriptase activity completely. This effect, that was confirmed later in the literature (25), was not due to complexation of divalent cations (Mn^{2+}) by the phosphate

Table 1. Feasibility of enzymatic detection of newly synthesized MuLV reverse transcriptase in oocytes.

• protein synthesis in oocytes	: 19–25 ng.h^{-1}/oocyte	(8)
• weight % of newly synthesized proteins in oocytes, injected with 40 ng/oocyte of mRNA from MuLV-infected cells, that is virus-related	: 0.1 %	(a)
• weight % of viral proteins that is RT	: 1 %	(40,41)
• amount of RT synthesized in oocytes injected with mRNA from MuLV-infected cells	: 0.2 pg.h^{-1}/oocyte	
• (^{3}H)TMP incorporation in the assay when a virus sample was tested	: 5 cpm.pg^{-1}	(b)
• (^{3}H)TMP incorporation in the assay expected when pure RT molecules would be tested	: 500 cpm.pg^{-1}	
• reverse transcriptase activity expected in 100 oocytes injected with 40 ng/oocyte of mRNA from MuLV-infected cells and incubated for 24 h	: 2.4×10^5 cpm	

a) Calculated from immunoprecipitation results using anti-MuLV serum.

b) See fore-going text; weight of virus based on protein amount.

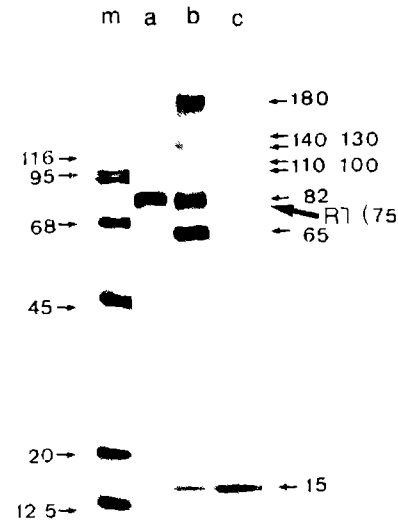

Figure 1. Characterization of the anti-RT serum. NIH-3T3(NCL5611P3)/Mo-MuLV cells were pulse-labeled for 15 min with (U-^{14}C)protein hydrolysate (lanes a,b) and radioactivity was chased for 8 h (lane c). Lysates were prepared and immunoprecipitation was carried out with anti-p15E,p12E (lane a) and anti-RT (lanes b,c). Precipitates were analyzed by SDS-PAGE followed by scintillation autoradiography. Lane m contains ^{14}C-labeled molecular weight marker proteins. Marker proteins were: cytochrome C (12,500), α-crystallin (20,000), ovalbumin (45,000), bovine serum albumin (68,000), phosphorylase a (95,000), and β-galactosidase (116,000)

Figure 2. (page 99)
RT-positive translation products made in oocytes on mRNAs from Mo-MuLV -infected cells.
Messenger RNA from NIH-3T3(NCL5611P3)/Mo-MuLV cells was isolated (100 /ug) and size-fractionated on an isokinetic sucrose gradient. Samples of each fraction were injected into 20 oocytes of Xenopus laevis. The oocytes were labeled for 20 h at 19 °C with L-(^{35}S) methionine in modified Barth's medium, lysates were prepared and immunoprecipitation was carried out with anti-RT. Precipitates were analyzed by SDS-PAGE followed by scintillation autoradiography. Positions of 18S and 28S ribosomal RNA on parallel gradients are indicated by big arrows. Small arrows indicate calculated S-values of RNA. Lanes p and c contain polypeptides that were precipitated from pulse and chase lysates of L-(^{35}S)methionine-labeled NIH-3T3(NCL5611P3)/Mo-MuLV cells. As a control, precipitates from lysates of oocytes injected with water are shown in lane "H_2O". Lane m contains ^{14}C-labeled marker proteins described in the legend to Fig. 1.

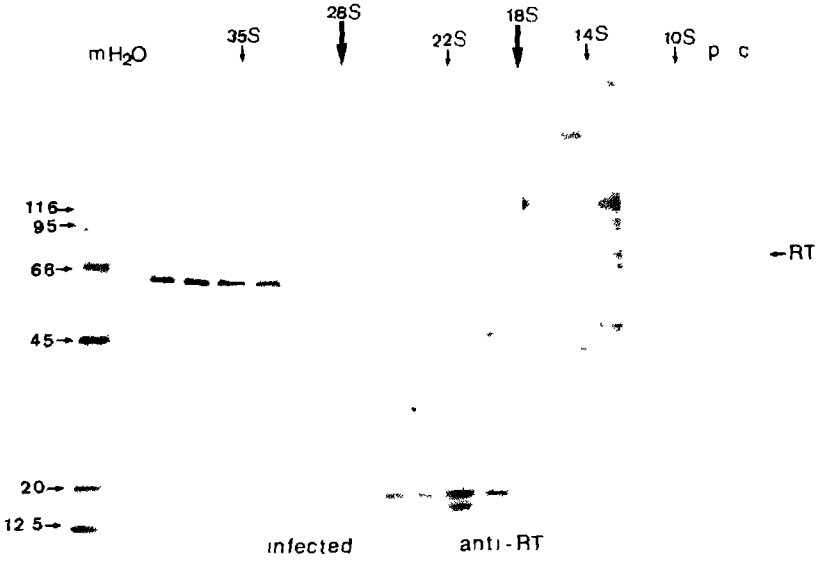

Figure 2. RT-positive translation products made in oocytes on mRNAs from Mo-MuLV -infected cells. (Legend on page 98).

ions since it could not be overcome by the addition of extra $MnCl_2$ (our own results and ref. 25). Instead of phosphate we used KCl (600 mM) to elute reverse transcriptase from oligo(dT)-cellulose. In the pilot experiments 90 % of the reverse transcriptase activity that we had added to oocyte lysates was recovered in the first four eluted fractions (total volume: 0.8 ml). We injected (unfractionated) mRNA from cells infected with wild-type R-MuLV (wt-248) and from cells infected with ts-29 into oocytes. Oocytes (80 - 120 in each experiment) were incubated for 16 h, 40 h or 64 h. By immunoprecipitation we found that viral gag and env proteins were synthesized (not shown). We were not able to detect any reverse transcriptase activity in the lysates of these oocytes, however. Since only relatively small samples of the high salt eluate from the oligo(dT)-cellulose column could be used in the assay, we attempted to concentrate the eluate by micro-ultrafiltration. These efforts failed, because, as we found in control experiments, reverse transcriptase activity was lost during this procedure. At this stage of our study an antiserum directed against Mo-MuLV reverse transcriptase became available to us (courtesy of Drs O.N. Witte and D. Baltimore).

Characterization of the anti-RT serum.

Mo-MuLV -infected cells were labeled with ^{14}C-labeled amino acids. On the lysates immunoprecipitations with anti-RT were performed. The precipitates were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). As shown in Fig. 1, lane b, after a 15 min pulse several polypeptides were precipitated. A double band with apparent molecular weight of 180,000 and bands at 140,000, 130,000, 110,000, 100,000, 82,000, 65,000, 30,000 and 15,000 are seen on the gel. The weak band at 15,000 is the gag protein p15 to which the antiserum is also directed (O.N. Witte, personal communication). The nature of the band at 30,000 is unknown; it is not the gag protein p30, since it is absent in the chase (Fig. 1, lane c), whereas p30 is present in much higher amounts in the chase than in the pulse (45). The band

at 65,000 is the gag precursor polyprotein $Pr65^{gag}$, precipitated by the contaminating anti-p15 antibodies in the antiserum. The band at 82,000 is presumably the envelope precursor polyprotein $gPr82^{env}$ (compare Fig. 1, lanes a and b) for reasons given in the next section. The higher molecular weight bands represent the precursor polyprotein to reverse transcriptase, $Pr180^{gag-pol}$, and its intermediary processing products (cf. ref. 16,18). After a chase of 8 h, the mature reverse transcriptase molecule with apparent molecular weight of 75,000 and the gag protein p15 are precipitated (Fig. 1, lane c).

Radioimmunoprecipitation of RT-positive translation products made in oocytes on size-fractionated mRNAs from MuLV-infected cells.

Messenger RNA was isolated from Mo-MuLV -infected NIH-3T3 cells by the magnesium-precipitation method of Palmiter (29), followed by selection on oligo(dT)-cellulose (3). In our hands this method was superior both in rapidity, yield and in translation efficiency of the isolated messenger RNA, compared to other methods, which included polyribosome isolation and/or phenol-extraction steps, used in our laboratory earlier (15,46). Messenger RNA was size-fractionated on isokinetic sucrose gradients and samples from each fraction were injected into Xenopus oocytes. Oocytes were labeled with L-(^{35}S)methionine. Immunoprecipitations were performed on the lysates. The results obtained by immunoprecipitation with antiserum directed against most of the MuLV proteins (anti-MuLV) are shown in chapter 5 (Fig. 1A) and chapter 6 (Fig. 3B). These results are similar to those obtained earlier (46, chapter 3): on mRNA of 35S size gag proteins (e.g. $Pr65^{gag}$ and p30) are synthesized and 22S mRNA directs the synthesis of the env proteins $gPr82^{env}$, gp70, p15E and p12E. Immunoprecipitations with anti-RT on the same lysates are shown here, Fig. 2. S-values of the injected mRNAs are indicated in the figure. As a reference, anti-RT immunoprecipitates from Mo-MuLV -infected cells, pulse-labeled for 15 min with L-(^{35}S) -methionine (lane p) and chased for 8 h (lane c), are shown. Note,

that p15 is missing in these precipitates, because p15 lacks methionine (45).

It can be seen that anti-RT precipitated polypeptides from lysates of oocytes injected with mRNAs of 15S, 22S and 35S size. The 200,000 molecular weight protein precipitated from the translation products of 15S mRNA was not further identified. A polypeptide of similar size was precipitated by anti-MuLV and anti-MCSA (Moloney cell surface antigen) (chapter 6, Fig. 3). As calculated in chapter 6, this 200,000 molecular weight protein is not a primary translation product of 15S mRNA, since mRNA of that size does not have sufficient coding capacity for such a large protein. The 22S products that were precipitated by anti-RT seem to represent the viral envelope proteins p15E and p12E. The results presented in Fig. 3 confirm this conclusion. It appeared that the 22S products precipitated by anti-RT (Fig. 3, lane b) comigrate with the products precipitable with anti-p15E,p12E (lane c) and that their precipitation could be inhibited by the addition of Mo-MuLV (lane a). This strongly indicates that the anti-RT serum contained contaminating antibodies not only directed against p15 but also against p15E, p12E. These latter antibodies presumably caused the precipitation of the 82,000 molecular weight protein by anti-RT from lysates of pulse-labeled cells (Fig. 1, lane b). From the lysates of oocytes injected with 35S mRNA a polypeptide of 65,000 apparent molecular weight, $Pr65^{gag}$, and the mature reverse transcriptase, p75, was precipitated by anti-RT. Therefore, it was concluded that the most characteristic component of RNA tumor viruses, the RNA-dependent DNA polymerase, is synthesized, at least in MuLV-infected cells, by genome-size messenger RNA.

DISCUSSION

Xenopus oocytes are well suited for translation studies. Messenger RNAs are remarkably stable in this system and, in contrast to cell-

Figure 3. Anti-p15E,p12E activity in the anti-RT serum.
Messenger RNA of 22S size from NIH-3T3 (NCL5611P3)/Mo-MuLV cells was injected into oocytes. Oocytes were labeled for 20 h with L-(^{35}S)methionine, lysates were prepared and immunoprecipitations were carried out. Precipitates were analyzed by SDS-PAGE followed by scintillation autoradiography. (a) Immunoprecipitation with anti-RT in the presence of 100 µg of added Mo-MuLV(clone 1A); (b) anti-RT; (c) anti-p15E,p12E.

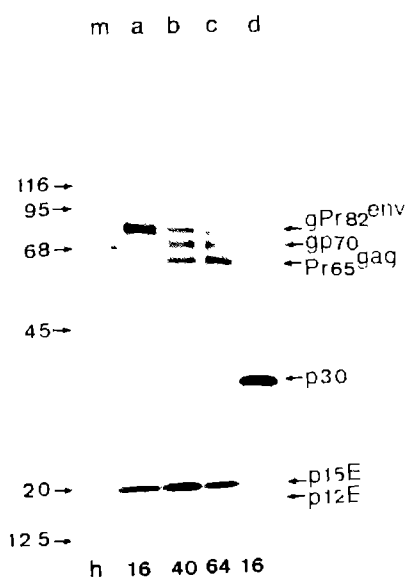

Figure 4. Translation kinetics of viral polypeptides in oocytes. Unfractionated mRNA from NIH-3T3(NCL5611P3)/Mo-MuLV cells (lanes a-c) and purified poly(A)$^+$ 35S viral RNA from clone 1A Mo-MuLV (lane d) were injected into oocytes. Oocytes were labeled for 16, 40 or 64 h (indicated at the bottom of each lane) with L-(^{35}S) methionine, lysates were prepared and immunoprecipitation was carried out with anti-R-MuLV. Precipitates were analyzed by SDS-PAGE followed by scintillation autoradiography. Lane m contains ^{14}C-labeled marker proteins (cf. Fig. 1).

free systems like those of rabbit reticulocytes or wheat germ, oocytes are not only able to translate exogenous mRNAs into their primary translation products, but these products are also processed to the final form in which the proteins are present in vivo. Post-synthetic modifications like proteolytic processing of pre- and polyproteins, glycosylation, phosphorylation, and N-terminal acetylation are faithfully carried out by the oocyte (for a review on this subject, see F.A.M. Asselbergs, thesis, Nijmegen, 1979). The oocyte seems to be the system of choice if one wishes to detect a translation product by its enzymatic activity, since a non-processed precursor protein may well be enzymatically inactive.

The purpose of the experiments described in this chapter was to establish the size of the pol-mRNA in a similar manner as the sizes of the gag-mRNA and env-mRNA were established previously (46; chapter 3). At the time we started these investigations the synthetic pathway of reverse transcriptase was not known (46; chapter 3) but soon it was shown that genome-size viral RNA directs the synthesis of minor amounts of polyproteins reactive both with antisera directed against gag proteins and with anti-reverse transcriptase antisera (23,27,30,33,52). Similar polyproteins were found in infected cells (16,18,53). These results suggest but do not rigorously prove that gag-pol polyproteins synthesized on genome-size virus-specific mRNA would also be the intracellular precursors to reverse transcriptase. A direct translation of intracellular virus-specific mRNAs would complete the evidence. For this purpose we developed a sensitive enzymatic assay to detect reverse transcriptase in oocyte lysates, since no antiserum to this viral protein was available to us at the time. Despite of all our efforts, however, we did not find any polymerase activity in oocytes injected with unfractionated mRNAs from infected cells. The mRNA preparation was active in translation, for gag and env (poly-)proteins were synthesized (not shown).

Variations of the incubation time of the injected oocytes and ef-

forts to concentrate the 600 mM KCl eluate from the oligo(dT)-cellulose column (to enlarge the fraction of the total potential RT activity recovered from the oocytes that could be tested in the enzyme assay) were unsuccessful. A number of explanations for the apparent lack of reverse transcriptase activity in this system are available. Failure of the oocyte system to synthesize reverse transcriptase or to process the primary (inactive) translation product, or to produce the enzyme in sufficient quantities for detection (due to breakdown of enzyme and/or mRNA) can be excluded, since we detected mature reverse transcriptase in oocyte lysates by immunoprecipitation (this method having about the same lower detection limit as the enzymatic assay).

Alternatively, one could imagine that mature reverse transcriptase molecules were made in oocytes, but in an inactive form. A third possibility is the inhibition of pol-mRNA translation by competition with the large excess of other mRNAs in the mixture containing all mRNA size-classes. Exogenous mRNAs in oocytes have to compete with each other and with the endogenous mRNAs for the translation machinery (2,22). The injected unfractionated mRNA samples contained more mRNA than the fractionated mRNA samples (see Methods section). Positive (5) and negative (3) reports have been published on the preference of the oocyte system for smaller mRNAs. From immunoprecipitation studies (see below) we know that reverse transcriptase is made on a large messenger. Supporting the hypothesis of competitive inhibition of pol-mRNA translation when unfractionated mRNA is injected into the oocyte, is our finding (Fig. 4) that the gag proteins were synthesized much slower when unfractionated mRNA was injected than when 35S mRNA was injected. In the latter case p30 is formed within 20 h (chapter 5, Fig. 1A and chapter 6, Fig. 3B), whereas in the former case p30 only emerged after more than two days of incubation (this chapter, Fig. 4, a-c). When highly purified 35S poly(A)$^+$ viral RNA was injected, p30 was formed even more quickly than with 35S infected cell mRNA (Fig. 4.d).

The anti-RT serum we received in a later stage of our study was not only directed against reverse transcriptase, but also against the gag protein p15. Therefore, it precipitated $Pr65^{gag}$ and p15 (Fig. 1). Apparently, anti-RT was also directed against p15E,p12E. In oocytes injected with 22S mRNA from MuLV-infected cells proteins were made that were precipitable with anti-RT and comigrated on the gel with p15E and p12E (Figs 2 and 3). Precipitation was inhibited by the addition of an excess of unlabeled MuLV (Fig. 3). From lysates of pulse-labeled MuLV-infected cells a protein of 82,000 apparent molecular weight was precipitated with anti-RT that comigrated on the gel with $gPr82^{env}$ (Fig. 1) and probably was identical to the envelope precursor polyprotein. We can not firmly explain, why anti-RT did not precipitate p15E and p12E from infected cells after a chase and $gPr82^{env}$ from lysates of injected oocytes. With anti-MuLV both $gPr82^{env}$ and p15E,p12E can be precipitated from infected cells as well as from oocytes injected with virus-specific mRNAs (chapter 6, Fig. 1,2,4B). We can only interprete this inconsistency by assuming an environmental influence (mouse cell or frog oocyte) on the kind of env antigenic determinants that are exposed.

The experiments illustrated in Fig. 2 clearly indicate the size of pol-mRNA: the immunoprecipitation results show that reverse transcriptase is made on genome-size (35S) mRNA. We were not able to strengthen this conclusion by tryptic peptide mapping, since the amount of precipitated reverse transcriptase was too low. Higher molecular weight precursor proteins to RT were not found in oocytes (Fig. 2). Presumably they are processed quickly to the mature enzyme in the oocyte. In contrast, only higher molecular weight polyproteins were found in translations of virion RNA in vitro by Murphy et al. (28) who recently translated mRNA from MuLV-infected cells in a mouse cell-free system. In this system, 35S intracellular RNA directed the synthesis of $Pr180^{gag-pol}$. By a much broader size-class of mRNAs $Pr65^{gag}$ was formed. A similar phenomenon is observed in our system (Fig. 2). Murphy et al.

(28) interpreted this as evidence that $Pr180^{gag-pol}$ and $Pr65^{gag}$ are synthesized on separate mRNAs. Since maximal synthesis of both $Pr180^{gag-pol}$ and $Pr65^{gag}$ seems to be directed by the same mRNA fraction this interpretation is rather speculative. In fact, the best indication that separate gag- and pol-mRNAs are involved is given by Philipson et al. (33) who showed that addition of yeast suppressor tRNAs to a cell-free translation of MuLV virion RNA enhanced the synthesis of the gag-pol polyprotein at the expense of $Pr72^{gag}$, but did not influence the synthesis of $Pr65^{gag}$. The exact mechanism by which the reverse transcriptase precursor protein is generated in vivo needs to be clarified further.

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5. A 14S MESSENGER RNA DIRECTING THE SYNTHESIS OF PRESUMABLY HOST-CODED PROTEINS PRESENT IN MURINE LEUKEMIA VIRUSES

A 14S MESSENGER RNA DIRECTING THE SYNTHESIS OF PRESUMABLY HOST-CODED PROTEINS PRESENT IN MURINE LEUKEMIA VIRUSES.

ABSTRACT

In chapter 3 a protein is described that was immunoprecipitated by an anti-MuLV serum from the Xenopus oocyte translation products of 14S messenger RNA isolated from Rauscher murine leukemia virus (R-MuLV) infected BALB/c cells. We extended this study by translating messenger RNAs from a different cell type (NIH-3T3) infected with a different virus (Moloney-MuLV) in the oocyte system. Similar results were obtained as before: a protein with an apparent molecular weight of 37,000 was precipitated by the anti-MuLV serum from lysates of oocytes injected with 14S mRNA. The 37,000 product was not precipitated by any mono-specific antiserum.

The same protein was synthesized in oocytes injected with 14S mRNA from uninfected NIH-3T3 cells. Therefore, the protein is presumably coded for by the host genome. Attempts to confirm this conclusion by hybrid-arrested translation with virus-specific DNA unfortunately failed, apparently due to the fragility of the mRNAs under hybridization conditions.

Other 14S mRNA translation products could be precipitated with an antiserum directed against Moloney-MuLV and an antiserum against Moloney cell surface antigen (MCSA). The nature of these 14S mRNA translation products that were shown to be present in virions will be discussed.

INTRODUCTION

In chapter 3 a model was proposed for the gene expression of murine leukemia viruses that consists of two separate messenger RNAs for the synthesis of gag and env proteins (35S mRNA and 22S mRNA, respectively). Moreover, we also considered the possibility of the existence of separate mRNAs for the synthesis of reverse transcriptase and of a putative onc protein (that would cause cell transformation and oncogenesis). At the time of that study (1976) the existence of a separate onc (or luk) gene of murine leukemia viruses (in analogy to the presence of a separate src gene in avian sarcoma viruses) was a speculation on which much research in this field was focussed. Although the absence of a separate onc gene in these viruses is not yet rigorously established, most experimental evidence (discussed in chapter 2 of this thesis) suggests a mechanism for viral leukemogenesis that is no longer based on this concept. Pol- and onc-mRNAs of different size classes were considered by us at the time because their existence would generalize the data obtained for the expression of the gag and env genes and because of the following suggestive indications. From the translation products of 25-28S mRNA a protein with apparent molecular weight of 85,000-90,000 and in the 14S region a 40,000-45,000 molecular weight protein was precipitated. Moreover, by molecular hybridization of size-fractionated JLS-V9/R-MuLV mRNA with copy-DNA prepared against R-MuLV viral RNA a peak at 14S was found in the hybridization profile. We could not detect a MuLV-specific mRNA of 25S size by molecular hybridization. A 14S mRNA was not detected by other investigators who have published on this subject (11,13), but its existence was reported for feline leukemia virus (8) and mouse mammary tumor virus (23,27) infected cells. In both cases weak (8) or strong (23) evidence indicated that the 14S mRNA was a specific degradation product of 35S RNA encompassing the 3' end of the genome. If a 14S virus-specific mRNA also would exist in

MuLV-infected cells, it certainly would not share a common 5' terminus with 35S and 22S virus-specific mRNAs (13,20,24).

In order to assess whether or not the immunoprecipitation of products of 25S and of 14S mRNA was restricted to a particular cell line or a single virus type, we isolated mRNA from Mo-MuLV -infected NIH-3T3 cells that subsequently was size-fractionated on isokinetic sucrose gradients and translated in oocytes of Xenopus laevis. The 25S mRNA product was not found, but anti-MuLV precipitated a 37,000 apparent molecular weight protein from the 14S mRNA translation products.
In the fore-going chapter it was shown that reverse transcriptase is not made on 25S mRNA but on genome-size mRNA. In this chapter the nature of the 14S mRNA product is further described.

MATERIALS AND METHODS

Materials. Radiochemicals and other materials used are described in the corresponding sections of chapters 4 and 6.
Antisera. Rabbit antisera against disrupted R-MuLV, (R-MuLV)p30 and (R-MuLV)p15E,p12E were prepared and described by Van Zaane et al. (34). Rabbit antisera against (R-MuLV)gp70 and (AKR)p10 were a gift of Dr J.N. Ihle, goat anti-(R-MuLV)p12 was a gift of Dr J.R. Stephenson, and goat anti-(R-MuLV)p15 was a gift of Drs M. Strand and J.T. August. Goat anti-Mo-MuLV was obtained through the Office of Program Resources and Logistics of NCI/NIH, Bethesda, Md. (Dr J. Gruber).
Cells and virus. NIH-3T3 cells (clone NCL5611P3) (32) were obtained from Dr J.R. Stephenson and infected with Mo-MuLV (cf. chapter 6). A recloned NIH-3T3 line was obtained from Dr R.A. Weinberg. Uninfected and Mo-MuLV -infected JLS-V9 cells (37) were obtained from Dr E.M. Fenyö.
Cells were grown in monolayer cultures in Dulbecco's modified

Eagle's medium with Earle's salts supplemented with 10 % newborn calf serum. R-MuLV was isolated from the plasma of viremic BALB/c mice. Mo-MuLV, isolated from roller-bottle cultures of NIH-3T3/Mo-MuLV (clone 1A) cells was a gift of Dr A.J.M. Berns.

Isolation and fractionation of mRNA and translation in Xenopus oocytes. Messenger RNA was isolated from Mo-MuLV -infected and from uninfected cells, size-fractionated and translated in oocytes of Xenopus laevis as described in chapter 4.

Immunoprecipitation and polyacrylamide gel electrophoresis. See corresponding section of chapter 4.

Hybrid arrested translation using in vitro synthesized copy-DNA. A single-stranded DNA-copy of Mo-MuLV vRNA was synthesized in an endogenous reverse transcription reaction with predigested calf thymus DNA (30) as a primer.

A reaction mixture of 1 ml contained: 50 mM Tris-pH 8.3, 3 mM $Mg(Ac)_2$, 60 mM NaCl, 20 mM dithioerythritol, 0.04 mM (0.5 mCi) (methyl-^{3}H)thymidine 5'-triphosphate, 1 mM each of dATP, dGTP and dCTP, 2 mg calf thymus DNA, 0.016 % Nonidet P-40, 75 /ug actinomycin-D and 1 mg virus. After incubation for 2.5 h at 37 ^{o}C, EDTA was added to 10 mM, SDS to 0.5 % and NaOH to 0.3 M (final concentration). The mixture was incubated overnight at 37 ^{o}C to degrade the RNA and was desalted on Sephadex G-50, using 10 mM Tris-pH 7.4, 1 mM EDTA (TE-buffer) as elution buffer. The DNA was phenol-extracted and alcohol-precipitated at neutral pH.

The DNA (26 /ug) was self-annealed for 3 h at 68 ^{o}C in 10 mM Tris-pH 7.4, 1 M NaCl, 1 mM EDTA ($c_o t$ = 2.75 Mol.sec.l^{-1}) and fractionated on a hydroxyapatite column (Bio-Gel HTP, Bio-Rad Labs, Richmond, Cal.) (3). Of the single-stranded DNA fraction 10 /ug was hybridized to 6 /ug of Mo-MuLV 35S viral RNA in 2 ml of 0.12 M sodium phosphate - pH 7.2, 0.2 % SDS. The viral 35S RNA was a gift of Mr C.J.M. Saris. It was isolated from Moloney clone 1A virus (12) as a 60-70S RNA-complex, oligo(dT)-selected and size-fractionated as described for cellular mRNA.

Hybridization was at 68 ^{o}C for 105 min (this was the time needed

to just reach the maximum level of hybridization, as determined by the S_1-nuclease procedure (16)). The hybrids were isolated by hydroxyapatite chromatography, dialyzed against TE-buffer and alcohol-precipitated with E. coli tRNA as a carrier. The RNA was hydrolyzed by incubation for 2 h at 50 °C in 0.3 M NaOH in TE-buffer and the DNA was alcohol-precipitated twice at neutral pH. Hybridization of an excess of cDNA to virus-specific mRNA was performed as described by Hastie and Held (15). In 5 /ul reaction mixtures 0.5 or 0.05 /ug of cDNA was hybridized to 1/ug of cellular (unfractionated) mRNA in 20 mM Hepes-pH 7.3, 180 mM KCl for 30 min at 65 °C ($c_o t$ = 0.625 or 0.0625 Mol.sec.l^{-1}, respectively). After quenching on ice samples were injected into oocytes. Controls consisted of mRNA alone subjected to the same procedure, mRNA.DNA mixtures heat-denatured for 30 sec at 100 °C after hybridization, non-hybridized mRNA.cDNA mixtures and mRNA alone injected into oocytes directly.

Hybrid-arrested translation using molecular cloned proviral DNA.
pBR322 plasmid with the proviral Mo-MuLV genome (large form; refs 14,38) integrated at the Hind III site (36) was obtained from the group of Dr D. Baltimore at the 1979 Cold Spring Harbor RNA Tumor Virus Course by Mr H. van der Putten from our department.
The recombinant-plasmid DNA was alkali-digested in 0.75 M NaOH, 0.5 mM EDTA for 15 min at 100 °C, and, after neutralizing the mixture with acetic acid, DNA was alcohol-precipitated. Three /ug of DNA was hybridized to 2 /ug of cellular NIH-3T3(NCLS611P3)/Mo-MuLV mRNA according to the procedure of Paterson et al. (21) in 80 % formamide, 15 mM Pipes-pH 6.4, 0.4 M NaCl and 2 mM EDTA. The mixture was heated for 30 sec at 68 °C and then incubated for 2 h at 48 °C. After hybridization the mixture was quickly chilled in CO_2/methanol and diluted 10-fold with ice-water containing 4 /ug of rat lens 28S rRNA.
After heat-denaturing half of the diluted hybridization mixture for 60 sec at 80 °C the nucleic acids of both fractions were alcohol-precipitated at pH 5.2.

Each fraction was dissolved in 2 µl of distilled water and samples were injected into oocytes. Controls consisted of mRNA alone subjected to the same procedure, mRNA and mRNA.DNA mixtures kept at 0 °C instead of being incubated at 48 °C and mRNA that was injected into oocytes directly.

RESULTS

Translation of size-fractionated mRNAs from Mo-MuLV -infected and uninfected NIH-3T3 cells in Xenopus oocytes.

Cellular messenger RNAs were isolated from Mo-MuLV -infected and from uninfected NIH-3T3(NCL5611P3) cells, size-fractionated on isokinetic sucrose gradients and samples of each fraction were injected into oocytes of the African clawed frog Xenopus laevis. As a control, H_2O was injected. After incubating the oocytes for 20 h in the presence of L-(^{35}S)methionine the oocytes were lysed and the extracts were subjected to immunoprecipitation with anti-R-MuLV followed by SDS-polyacrylamide gel electrophoresis (Figs 1A, 1B; note, that Figure 1A is identical to Figure 3B in chapter 6). S-values of the injected mRNAs are indicated at the top of the figures. The gel analysis of the immunoprecipitate formed by anti-R-MuLV with lysate of H_2O-injected oocytes serves to indicate which protein bands were due to aspecific precipitation. Fig. 1A shows the anti-R-MuLV precipitates obtained from lysates of oocytes injected with mRNA from Mo-MuLV -infected cells; the immunoprecipitable products of mRNAs from uninfected cells are shown in Fig. 1B. The results presented in Fig. 1A are similar to those reported and discussed by us before (35; chapter 3), except that we were not able to precipitate a 85,000-90,000 molecular weight protein from the translation products of 25S mRNA. The gag (poly)proteins $Pr65^{gag}$ and p30 were synthesized on genome-size (35S) mRNA. The lower molecular weight structural core proteins can not be seen, since p15 and p10 lack methionine and p12 is not recog-

Figure 1. (A + B) (page 121).
Virus-related translation products made in oocytes on mRNAs from Mo-MuLV -infected and uninfected cells.
Messenger RNA from Mo-MuLV -infected and uninfected NIH-3T3 cells was isolated and size-fractionated on isokinetic sucrose gradients. Samples of each fraction were injected into 20 oocytes of Xenopus laevis. After labeling the oocytes for 20 h at 19 °C with L-(^{35}S) methionine in modified Barth's medium, immunoprecipitation was carried out on the lysates with anti-R-MuLV. Precipitates were analyzed by SDS-PAGE followed by scintillation autoradiography.
(A) Messenger RNA from Mo-MuLV -infected cells; (B) mRNA from uninfected cells. Positions of 18S and 28S ribosomal RNA on parallel gradients are indicated by big arrows. Small arrows indicate calculated S-values of RNA.
Lanes p and c (B) contain polypeptides that were precipitated from pulse and chase lysates of L-(^{35}S)methionine labeled NIH-3T3 cells, in lane "p+c" (A) precipitated polypeptides from combined pulse and chase lysates of NIH-3T3/Mo-MuLV(clone 1A) cells are shown (cf. chapter 6). As a control, precipitates from lysates of oocytes injected with water are shown in lanes "H_2O". Lanes m contain ^{14}C-labeled molecular weight markers. Marker proteins were: cytochrome c (12,500), α-crystallin (20,000) ovalbumin (45,000), bovine serum albumin (68,000), phosphorylase a (95,000) and β-galactosidase (116,000).

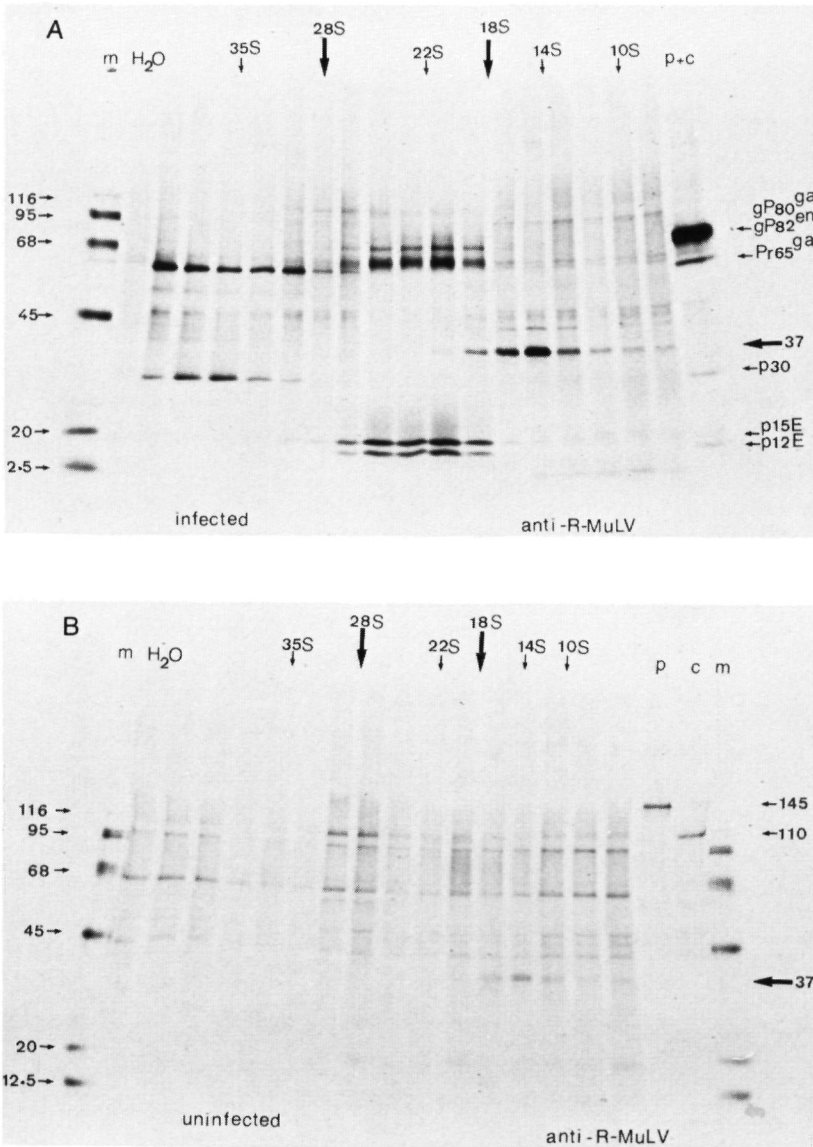

Figure 1. (A + B). Virus-related translation products made in oocytes on mRNAs from Mo-MuLV -infected and uninfected cells. (Legend on page 120).

nized by the antiserum used (34). The gag polyproteins $gP80^{gag}$, $gP95^{gag}$, and $gP85^{gag}$ are not found in oocytes after labeling periods longer than 10 h (25,35; F.A.M. Asselbergs, thesis, Nijmegen, 1979). Presumably these products are synthesized on a relatively unstable mRNA and have a rapid turnover. On 22S mRNA the env (poly-)proteins $gPr82^{env}$, gp70, p15E, and p12E were made. In the 14S region a protein with an apparent molecular weight of 37,000, tp37[1)], that was immunoprecipitable by anti-R-MuLV was synthesized. The molecular weight that we have calculated for this protein is slightly lower than we reported earlier for a similar protein synthesized on 14S mRNA from R-MuLV -infected JLS-V9 cells (35; chapter 3), yet both proteins migrate to the same positions when run on parallel lanes in a SDS-polyacrylamide gel (not shown). When mRNAs from uninfected NIH-3T3 cells (or uninfected JLS-V9 cells; not shown) were translated in oocytes, only the 37,000 molecular weight product of 14S mRNA was precipitable by anti-R-MuLV (Fig. 1B). Therefore, tp37 is presumably a host-coded protein.

Analysis of the 37,000 molecular weight product of 14S mRNA with mono-specific antisera.

Messenger RNA of 14S size from NIH-3T3(NCL5611P3)/Mo-MuLV cells was translated in oocytes and immunoprecipitations were carried out on the oocyte lysate with antisera directed against the gag proteins p30 (Fig. 2, lane a), p15 (lane c), p12 (lane d) and p10 (lane f), with antisera directed against the env proteins gp70 (lane b) and p15E,p12E (lane e), and with an anti-Mo-MuLV serum (lane g). Only anti-Mo-MuLV precipitated a protein of 37,000 molecular weight. Anti-gp70 weakly precipitated a protein with

1) To simplify notation, but avoiding the accepted nomenclature for retroviral proteins, this product is called tp37, denoting a translation product of 37 thousand molecular weight.

apparent molecular weight of 40,000. This protein was maximally produced by 28S mRNA, however (not shown). Faintly, p15E and p12E can be seen in lane e (immunoprecipitation with anti-p15E,p12E). Apparently (compare with Fig. 1A), resolution of the isokinetic sucrose gradient system was not very high. Anti-p12 and anti-Mo-MuLV precipitated several products of 14S mRNA (lanes d and g), some of which comigrated on the gel. The nature of these products is not known. Anti-p12 and anti-Mo-MuLV were completely mono- or virus-specific when lysates of virus-infected cells were used for immunoprecipitation (not shown).

From these results we concluded that the 37,000 molecular weight protein synthesized on 14S mRNA from MuLV-infected or uninfected cells can only be precipitated by antisera directed against complete MuLV.

Hybrid-arrested translation.

In order to confirm the conclusion that tp37 is not coded for by the virus we performed hybrid-arrested translations (15,21). Messenger RNA to be translated was pre-hybridized to proviral DNA. If the genetic information for tp37 forms an integral part of the viral genome, the translation of 14S mRNA into this protein would be inhibited by hybrid formation. If not, translation would be unimpaired. Two methods of hybrid-arrested translation were followed. First, a single-stranded copy-DNA, representing the complete genome in equal amounts, was prepared by reverse transcription in the endogenous system in the presence of actinomycin D and using nuclease-digested calf thymus DNA as a primer (30). Minor double-stranded pieces of DNA were removed by self-annealing followed by hydroxyapatite selection. Possible contaminations with cellular sequences were eliminated by hybridization of the cDNA against highly purified poly(A)$^+$ Mo-MuLV 35S viral RNA, followed by hydroxyapatite chromatography. Hybridization of the purified cDNA against (unfractionated) mRNA from Mo-MuLV -infected NIH-3T3 cells was performed under conditions that enabled the injection of the

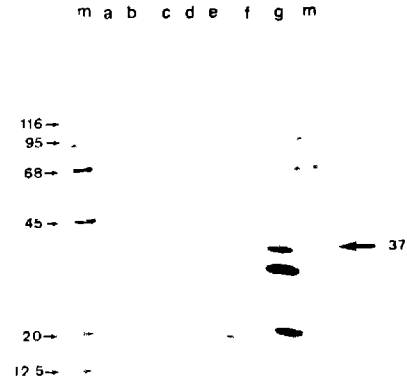

Figure 2. Analysis of 14S mRNA translation products with monospecific antisera.
Messenger RNA of 14S size from NIH-3T3(NCL5611P3)/Mo-MuLV cells was translated in oocytes. Translation products were analyzed by immunoprecipitation with: (a) anti-p30; (b) anti-gp70; (c) anti-p15; (d) anti-p12; (e) anti-p15E,p12E; (f) anti-p10 and (g) anti-Mo-MuLV, followed by SDS-PAGE and scintillation autoradiography. Lanes m contain ^{14}C-labeled molecular weight marker proteins described in the legend to Fig. 1.

Figure 3. (page 125).
Hybrid-arrested translation.
Unfractionated mRNA from NIH-3T3(NCL5611P3)/Mo-MuLV cells was hybridized to highly purified single-stranded copy-DNA of Mo-MuLV viral RNA and a sample of the hybridization mixture was injected into oocytes (see Methods section). (a) 1 /ug/5 /ul mRNA hybridized to 0.05 /ug cDNA; (b) 1 /ug/5 /ul mRNA hybridized to 0.5 /ug cDNA; (c) 1 /ug/5 /ul RNA mock-hybridized; (d) same as (a) but heated after hybridization for 30 sec at 100 ^{o}C to melt the hybrids; (e) same as (b) but with hybrids melted; (f) same as (c) but with "hybrids" melted; (g) 1 /ug/5 /ul mRNA mixed with 0.5 /ug cDNA, non-hybridized; (h) 1 /ug/5 /ul mRNA, non-hybridized; (i) 0.5 /ug/5 /ul cDNA, non-hybridized; (j) hybridization buffer; (k) H_2O. Lanes m contain ^{14}C-labeled molecular weight marker proteins (cf. Fig. 1.).

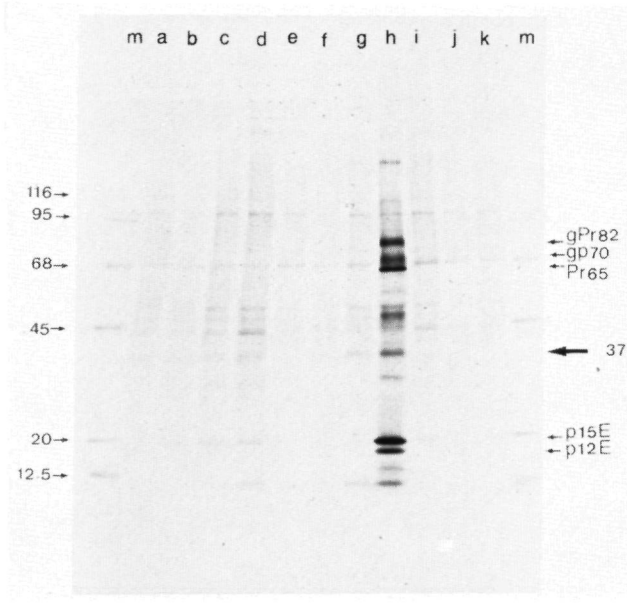

Figure 3. Hybrid-arrested translation. (Legend on page 124).

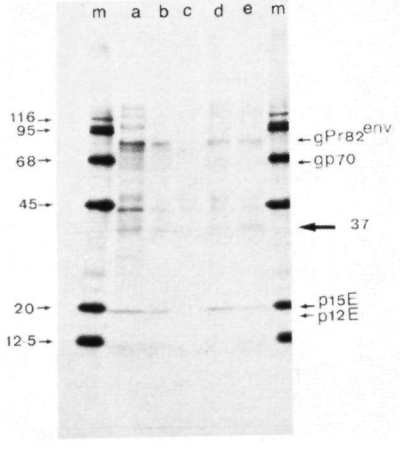

Figure 4. Presence of p37 in cells, serum, and virus. Messenger RNA of 16-18S size from NIH-3T3(NCL5611P3)/Mo-MuLV cells was injected into oocytes. On the lysates immunoprecipitations were carried out with anti-R-MuLV in the presence of added: (a) none; (b) 10 µg p30; (c) 100 µg Mo-MuLV; (d) 200 µl of a concentrated (10^7 cells/ml) lysate of NIH-3T3 cells; (e) 25 µl of newborn calf serum. Lanes m contain ^{14}C-labeled molecular weight marker proteins (cf. Fig. 1.).

complete mixture into oocytes directly after finishing the hybridization (15). The results of these experiments are shown in Fig. 3. It can be seen that the hybridization procedure caused a functional inactivation of the mRNA. After hybridization with cDNA (lanes a,b) or after mock-hybridization (lane c) no translational activity of the virus-specific messengers was left. This activity was not restored by melting the hybrids (lanes d - f). This indicates that the mRNA was broken down. On the other hand, a non-hybridized mixture of cDNA and mRNA also did not synthesize virus-specific translation products (lane g), except for tp37 (compare with lane h; in fact this protein is also found as a faint band in lanes a - d). The presence of tp37 in this case is not due to an artifact caused by the cDNA or the hybridization buffer (lanes i - k). If this would mean that the translation of the virus-specific messengers was inhibited by cDNA without hybridization, the conclusion would be that tp37 is not coded for by the viral genome. Although unlikely, on the basis of these results we can not totally exclude, however, that traces of RNAse activity have caused the break-down of the larger gag and env mRNAs without destroying the smaller 14S mRNAs completely.

Hybridization in the presence of 50 % or more formamide subjects the mRNA to milder conditions since hybridization can be carried out at lower temperatures (3), but it necessitates an extra alcohol-precipitation step before the injection of the hybridization mixture into oocytes.

Despite of the selection procedure, the cDNA probe could theoretically still have contained cellular sequences. Molecular-cloned Mo-MuLV DNA, on the other hand, is absolutely free of cellular sequences. In order to prevent the double-stranded cloned Mo-MuLV DNA sequences to hybridize with themselves, special hybridization conditions had to be chosen that favoured the formation of RNA.DNA hybrids (19,22). This hybridization procedure (see Methods section) with the cloned DNA probe was successfully used by Mr C.J.M. Saris in our laboratory for hybrid-arrested translation of viral RNA in

the nuclease-treated reticulocyte cell-free system. Unfortunately, these milder hybridization conditions still caused the loss of the mRNA translational activity in oocytes completely (not shown).

Presence of tp37 in cells, serum and virus.

The 37,000 molecular weight protein was made on 14S cellular mRNA in oocytes, but was not detected in lysates of labeled, infected or uninfected, cells (chapter 6, Fig. 2). It was only precipitated by antisera directed against complete MuLV (this chapter, Figs 1 and 2). Therefore it seemed logical to assume that tp37, or a related protein, was present in the virions against which the anti-R-MuLV serum was raised. These virions, isolated from infected mice, might have contained a cellular or a serum protein that cross-reacts with a protein present in calf serum. Since the cellular lysates contained calf serum (see Methods section), this would explain why we were not able to find tp37 on autoradiograms of gels run with anti-R-MuLV precipitates from lysates of labeled mouse cells. To test this hypothesis, competition experiments were performed. The anti-R-MuLV precipitation was carried out on lysates of oocytes injected with 16-18S mRNA from Mo-MuLV -infected NIH-3T3 cells. From these lysates both env-proteins and tp37 could be precipitated (Fig. 4, lane a; compare with Fig. 1A). By this means an internal control on the function and specificity of the competition system was established. An other control of the specificity was achieved by competition with p30. This protein should not compete with tp37 or the env proteins. This indeed appeared not to be the case (Fig. 4, lane b). On the other hand, R-MuLV isolated from the plasma of viremic BALB/c mice competed with the env-proteins as well as with tp37 (lane c). The virus preparation used in this experiment was similar to the one used for raising the anti-R-MuLV serum (33,34). Moreover, Mo-MuLV isolated from roller-bottle cultures of infected NIH-3T3 cells (clone 1A) also competed with tp37 (chapter 6, Fig. 6). Apparently, calf serum nor a lysate of uninfected NIH-3T3 cells contained the 37,000 molecular weight

protein (or a cross-reactive activity) (Fig. 4, lanes d,e).
We conclude from these results that tp37 is a, presumably host-coded, virion protein that has no counter part in calf serum and that is not present in substantial amounts in lysates of mouse fibroblasts.

DISCUSSION

Not only 14S mRNA from JLS-V9 cells (35; chapter 3), but also mRNA of the same size from NIH-3T3 cells (this chapter, Fig. 1) directs the synthesis in oocytes of a 37,000 molecular weight protein precipitable with anti-MuLV serum.
It is tempting to speculate on a putative onc-gene origin of tp37, comparing this product with the src gene-product of avian sarcoma virus (ASV). The 3' end of the ASV genome contains a genetically and biochemically defined gene, called src, that is responsible for the sarcomagenic activity of the virus in vivo and its cell-transforming capacity in vitro (for a review: ref. 2 and chapter 2.4.). The product of this gene, $pp60^{src}$, is a 60,000 molecular weight phosphoprotein with cAMP-independent protein kinase activity (5, 10, 17), that originally was found by immunoprecipitation with a serum from ASV-induced tumor-bearing rabbits in lysates of ASV-transformed chicken and hamster cells (4). This protein did not cross-react with any of the viral structural proteins, and, initially, was not found in normal cells, in cells infected with a transformation-defective (td) strain of ASV or in cells infected at the non-permissive temperature with an ASV mutant having a temperature-sensitive expression of its transforming capacities. Mainly therefore it was considered as the product of the src-gene (4).
This conclusion was amply confirmed by in vitro translation of the 3' one-third (22S) of the ASV genome (1, 10, 22). It soon appeared, however, that a 60,000 molecular weight phosphoprotein with protein kinase activity, closely related to the src-gene product, was

present in normal cells, though at lower levels than in ASV-transformed cells (6,7). This was in accordance with the finding of src-related mRNA in normal cells (28,29).

The most striking difference between the expression of the avian src-product and that of the murine 14S mRNA 37,000 molecular weight product is the absence of the latter protein in MuLV-infected or uninfected mouse fibroblasts. One should bear in mind, however, that fibroblasts are not the target cells for leukemia viruses and that these cells can not be transformed by MuLV (2). It would be conceivable that the expression of a putative onc-gene -related protein is repressed in fibroblasts. This would explain why tp37 was not precipitated from lysates of labeled, infected or uninfected, fibroblasts and why immunoprecipitation of tp37 formed in oocytes could not be inhibited by the addition of a concentrated extract of unlabeled NIH-3T3 cells (Fig. 4, lane e). The repression should act on the level of translation, since mRNA able to synthesize tp37 is present in the fibroblast cells (Figs 1A and 1B).

The src-protein is not present in ASV particles (4), but tp37 or a related protein is present in R-MuLV and Mo-MuLV virions (Fig. 4, lane c and chapter 6, Fig. 6). The presence of tp37 in Mo-MuLV particles is hard to explain if one assumes that tp37 is not expressed in fibroblasts, since Mo-MuLV was grown in NIH-3T3 fibroblast cells (12). Although the lysates might have lacked some nuclear constituents which therefore escaped detection by immunoprecipitation or competition, it is hardly conceivable that a nuclear cell protein would enter the virions.

The 37,000 molecular weight protein is not a serum protein aspecifically adsorbed to the virus particles, since it did not compete with calf serum (Fig. 4, lane d). The complete unrelatedness to other known structural virus proteins is a common feature of $pp60^{src}$ and tp37. We labeled oocytes with ^{32}P-phosphate, but this kind of protein-labeling is - for unknown reasons - very inefficient in oocytes (Dr W.J.M. van de Ven, personal communication) and we were not able to detect phosphoproteins (not even without immunoprecipi-

tation) (not shown). Therefore it could not be assessed whether tp37 is a phosphoprotein like pp60^{src} or not.

The src-related mRNA of normal chicken cells is not of the same size as the virus-specific src-mRNA (28,29). In the murine case tp37 is synthesized on 14S mRNA both from MuLV-infected and from uninfected cells. The viral src-mRNA has its 5' terminus in common with the gag- and env-mRNAs (9,19). The MuLV-specific 14S RNA found by hybridization (35; chapter 3) might either be the product of an artifact or a degradation product of genome-size RNA not derived by a splicing mechanism in which the genomic 5' terminus is conserved (8,11,13,20,23,24,27,35). The anti-Mo-MuLV serum not only precipitated tp37, but also, even more strongly, proteins with apparent molecular weights of 33,000, 24,000 and 22,000.

The immunoprecipitation of these proteins could also be blocked by the addition of Mo-MuLV virions (not shown). Finally, anti-MCSA, an antiserum raised against a Mo-MuLV -induced lymphoma in syngenetic mice, precipitates still other protein products of 14S mRNA that cross-react with proteins present in Mo-MuLV virions, but that could not be precipitated from infected or uninfected fibroblasts (chapter 6).

The nature of all these 14S mRNA products still remains obscure. The circumstance that all these products are made on mRNA of the same size and are encapsidated into virions, whereas messengers of different size did not give rise to the production of such non-structural virion proteins may have a significance that is not yet understood.

In summary, we conclude that tp37 and other products formed in oocytes of Xenopus laevis upon injection with 14S mRNA from MuLV-infected or uninfected mouse fibroblasts are identical to or cross-react with proteins present in MuLV virions.

Because of our failure to perform hybrid-arrested translations, we can not exclude rigorously that tp37 is coded for by the viral and by the cellular genome, as is the case with pp60^{src} of ASV. Yet the

present state of our knowledge of the genetic organization of the murine leukemia viruses does not favour that possibility.

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6. MOLONEY CELL SURFACE ANTIGEN (MCSA) IS AN ENV-GENE PRODUCT THAT IS SEROLOGICALLY DISTINCT FROM THE ENV-GENE PRODUCTS OF THE MOLONEY STRAIN OF MURINE LEUKEMIA VIRUS.

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MOLONEY CELL SURFACE ANTIGEN (MCSA) IS AN ENV-GENE PRODUCT THAT IS SEROLOGICALLY DISTINCT FROM THE ENV-GENE PRODUCTS OF THE MOLONEY STRAIN OF MURINE LEUKEMIA VIRUS.

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ABSTRACT

Moloney cell surface antigen (MCSA) is serologically detectable on Moloney murine leukemia virus (Mo-MuLV) -induced lymphomas. It is probably related to the tumor-associated transplantation antigen of these lymphomas.

We characterized MCSA by using the typing antiserum in radioimmunoprecipitations followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. For comparison, immunoprecipitations with antisera directed against viral proteins were also carried out.

Surface-iodinated Mo-MuLV -infected cells were used to identify the molecular nature of MCSA. It appeared that MCSA was expressed as a single entity of 82,000 apparent molecular weight ($p82^{MCSA}$). The biosynthesis and turnover of this molecule, that comigrated on the gel with $gPr82^{env}$, was studied with metabolically pulse-chase labeled cells. It was found that $p82^{MCSA}$ had a high turnover but no immunoprecipitable processing products were found. Translation of size-fractionated mRNAs from infected cells in oocytes of *Xenopus laevis* showed that $p82^{MCSA}$ was made on a mRNA of the same size (22S) as that coding for the Mo-MuLV *env* proteins. Moreover, $p82^{MCSA}$ was processed in oocytes to proteins exactly comigrating on the gel with gp70, p15E and p12E.

Competition experiments with unlabeled virus and viral proteins showed that MCSA was not related to any of the Mo-MuLV structural proteins.

We concluded that MCSA is an *env* gene product presumably derived from a contaminating defective type-C virus present in the Mo-MuLV isolates as a pseudotype.

INTRODUCTION

Moloney cell surface antigen (MCSA) is present on lymphomas induced by Moloney murine leukemia virus (Mo-MuLV) in newborn mice (Klein and Klein, 1964; Klein et al., 1966).
The typing antiserum is raised against an irradiated Mo-MuLV -induced lymphoma (YAC) of A mouse origin in $(A \times C57Bl)F_1$ or $(A \times C57L)F_1$ mice (Klein et al., 1966).
MCSA is not present on normal lymphoid cells but can be found on some Mo-MuLV or Rauscher murine leukemia virus (R-MuLV) -infected fibroblast cell lines (Fenyö and Klein, 1976; Grundner et al., 1974; Kurth et al., 1979). It seems to be closely related to the tumor-associated transplantation antigen (TATA) of Mo-MuLV -induced lymphomas, since the amount of MCSA present on tumors correlates quite well with the immunosensitivity of the tumors in vivo, as measured by the rejection of small tumor isografts in preimmunized mice (Klein et al., 1966).
Although YAC lymphoma cells, used to raise the anti-MCSA serum, express viral proteins on their surface (Fenyö and Klein, 1976; Fenyö et al., 1977; Karande et al., 1979) and the anti-MCSA serum contains some antibody activity against gp70 (Fenyö et al., 1977), several lines of evidence indicate that the main activity in the antiserum is not directed against antigen(s) present on structural proteins of Mo-MuLV. First, it was found that two sublines of the YAC line that were made immunoresistant to the anti-MCSA serum by immunoselection in vivo and in vitro did not express MCSA anymore, but were as sensitive as the parental YAC line to the cytotoxic effect of monospecific hetero-antisera against structural proteins of the Friend-Moloney-Rauscher subgroup of MuLV (Fenyö and Klein, 1976). Second, the cytotoxic effect of anti-MCSA to the YAC cells could not be inhibited by viral proteins or virus of the FMR subgroup (Fenyö et al., 1977). Finally, by chromatography (Siegert et al., 1977) as well as by electrophoresis (Troy et al., 1977) MCSA

activity (measured by inhibition of anti-MCSA directed cytotoxicity) could be separated from viral structural proteins and H-2 antigens. On the other hand, though MCSA did not co-cap with the exogenous viral envelope proteins gp70 and p15E in membrane immunofluorescence (Fenyö et al., 1977), it did so with the viral core proteins p30 and p12 (Karande et al., 1979). Moreover, on a lectin affinity column p15 partially co-chromatographed with MCSA (though p15 itself is not glycosylated) (Siegert et al., 1977) and on an anti-p15 immunosorbens column part (maximally 15 %) of the MCSA activity was bound (Karande et al., 1979).

Therefore, it was suggested (Karande et al., 1979), that MCSA resembles the feline oncorna-virus associated cell membrane antigen (FOCMA) (Stephenson et al., 1977) in that it is synthesized as a polyprotein of p15 (and p12?) covalently linked to a non-structural protein. This polyprotein would be further processed to the mature, non-gag-linked MCSA molecule, that could become losely associated with the gag proteins on the cell surface.

We characterized MCSA by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of immunoprecipitates obtained with anti-MCSA and antisera against viral structural proteins from lysates of surface-labeled and metabolically labeled cells and of Xenopus laevis oocytes injected with size-fractionated cellular messenger RNAs.

The results confirm that MCSA is not serologically related to any of the Moloney virus proteins. On the other hand, they also indicate that anti-MCSA recognizes env gene-products of some other type-C virus or viruses.

MATERIALS AND METHODS

Materials. L-(^{35}S)methionine (spec. act. 800-1200 Ci/mmol), (U-^{14}C)protein hydrolysate (spec. act. 54 mCi/matom carbon), D-(1-^{3}H)glucosamine (spec. act. 54.1 Ci/mmol) and iodine-125

(carrier-free) were obtained from the Radiochemical Centre, Amersham, England. Iodo-Gen (1,3,4,6-tetrachloro-3α,6α -diphenyl-glycoluril) was purchased from Pierce Chem. Co., Rockford, Ill. Oligo(dT)-cellulose (T-2) was from Collaborative Research Inc., Waltham, Mass. Heparin (sodium salt, medical grade, 160 U/mg) was purchased from Organon, Oss, The Netherlands. Proteinase K was from Boehringer, Mannheim, West-Germany. RNAse A and DNAse I (both purest grade) were from Sigma, St. Louis, Mo. Protein A-Sepharose CL-4B was from Pharmacia, Uppsala, Sweden. (R-MuLV)gp70 was a gift of Dr J.R. Stephenson.

Antisera. The anti-MCSA serum was a gift of Dr E.M. Fenyö. Rabbit antisera against disrupted R-MuLV, (R-MuLV)p30 and (R-MuLV)p15E, p12E were prepared and described by Van Zaane et al. (1976). Rabbit-anti-(R-MuLV)gp70 was a gift of Dr J.N. Ihle. Ascites fluids containing monoclonal antibodies directed against six different env antigenic determinants (Lostrom et al., 1979) were a gift of Dr R.C. Nowinski. FITC-conjugated goat antiserum against mouse immunoglobulins was purchased from Nordic Immunological Labs., Tilburg, The Netherlands.

Cells and viruses. Recloned NIH-3T3 cells (Todaro and Green, 1963) were a gift of Dr R.A. Weinberg. The uninfected and Mo-MuLV -infected JLS-V9 cells (Wright et al., 1967) were a gift of Dr E.M. Fenyö. Two Mo-MuLV infected NIH-3T3 lines were used: one was the subclone A of the clone no. 1 cells described by Fan and Paskind (1974) and given to us by Dr H. Fan; the other was the clonal NIH-3T3 line NCL5611P3 (a gift of Dr J.R. Stephenson) infected with Mo-MuLV obtained from the JLS-V9/Mo-MuLV cells. Mink lung cells (CCL 64) infected with NZB xenotropic virus, AKR virus-infected NIH-3T3 cells (clone 56-n) and (AKR)MCF-247 virus-infected SC-1 cells were a gift of Dr F.C. Jensen to Dr A.J.M. Berns in our laboratory.

Cells were grown in monolayer cultures in Dulbecco's modified Eagle's medium with Earle's salts supplemented with 10 % newborn calf serum.

Moloney murine leukemia virus (Mo-MuLV) was isolated from concentrated medium of roller bottle cultures of clone 1A cells as described by Duesberg and Robinson (1966) and was given to us by Dr A.J.M. Berns. These virus preparations contained relatively low amounts of envelope proteins, presumably because of the partial loss of the viral envelope by shear forces during the concentration (ultrafiltration) of the medium.

Membrane immunofluorescence. Cells were plated in the wells (6 mm diameter) of Flow multitest slides (Flow Labs Ltd., Irvine, England). At about 50 % confluency the cells were washed three times by gently shaking the slides with Tyrode's balanced salt solution containing 0.1 % sodium azide to prevent cap formation. Subsequently, 20 µl of the antiserum (anti-MCSA) or the control serum (normal BALB/c or NIH Swiss mouse serum, depending on the cell line used) was added in a 1 : 40 dilution; incubation was for 30 min at room temperature; cells were washed three times and 20 µl of 1 : 80 diluted FITC-conjugated goat-anti-mouse (IgG) was added. After another 30 min incubation, the cells were washed six times, covered with a cover slip and examined with the aid of a Leitz Ortholux II immunofluorescence microscope.

Cell surface labeling and preparation of lysates. Cells were *surface-iodinated using solid phase-bound* 1,3,4,6-tetrachloro-3α, 6α-diphenylglycoluril as the iodinating agent (Markwell and Fox, 1978). Monolayer cells in 50 cm^2 glass culture flasks grown to subconfluency were rinsed eight times with Hanks' balanced salt solution (HBSS) and finally overlaid with 2.5 ml of HBSS to which 1 mCi of ^{125}I was added. Chloroglycoluril was dissolved in chloroform and a 25 mm diameter glass fiber filter was impregnated with 100 µg of the iodinating reagent. The filter was dried, rinsed with HBBS and placed at one end in the culture flask. Labeling was continued for 30 min and finished by removing the filter. Cells were rinsed eight times with HBSS containing 10 % calf serum, finally overlaid with 4 ml of this medium, and lysed by the addition of 1 ml of fivefold concentrated immunoprecipitation buffer (see

below). Lysates were incubated for 18 h at 6 °C after the addition of 200 µg/ml of RNAse A, 10 µg/ml of DNAse I, 5 µl/ml of normal rabbit serum and 1.0 ml/ml of a 10 % (v/v) suspension of Protein A-Sepharose CL-4B and clarified by centrifugation for 30 min at 200,000 x g (Van de Ven et al., 1980).

Metabolic cell labeling and lysis procedure. Cells were labeled with L-(^{35}S)methionine, (U-^{14}C)protein hydrolysate or D-(1-^{3}H) glucosamine according to the methods published by Van Zaane et al. (1976), with small modifications as described earlier (Van de Ven et al., 1977). Lysates were prepared by the addition of 1/4 volume of fivefold concentrated immunoprecipitation buffer (see below) to cells and medium and were cleared by centrifugation at 220,000 x g for 15 min.

Isolation and fractionation of mRNA. Cells were grown to 80 - 90 % confluency, removed from the culture flasks by trypsinization and washed twice with isotonic buffer (10 mM Tris-pH 7.2, 146 mM NaCl). Cellular RNA was isolated from these cells by the magnesium-precipitation method of Palmiter (1974). The precipitated RNA was dissolved in 10 mM Tris-pH 6.9, 20 mM NaCl, 10 mM EDTA and 0.5 % SDS to a concentration of 1 - 2 mg/ml and treated with preincubated proteinase K (100 µg/ml) for 15 min at 37 °C. The solution was then heated for 2 min at 100 °C, chilled, brought to 0.5 M NaCl final concentration and applied on an oligo(dT)-cellulose column. mRNA was collected by the method of Aviv and Leder (1972), except that we used NaCl instead of KCl.

The mRNA (50 - 150 µg) was fractionated according to size by centrifugation for 6.5 h at 40,000 rpm and 20 °C in an IEC SB-283 rotor on a 15 - 37.5 % (w/w) isokinetic sucrose gradient (Van der Zeijst and Bloemers, 1976) in low salt buffer (20 mM NaAc-pH 5.2, 2 mM EDTA, 0.2 % SDS). Prior to centrifugation the RNA was denatured in low salt buffer containing 85 % formamide by incubation for 5 min at 37 °C and 30 sec at 68 °C. Fractions (0.5 ml) from the gradient were collected and alcohol-precipitated after the addition of 10 µg rat lens 28S ribosomal RNA as a carrier.

Translation of mRNA in Xenopus oocytes. The mRNA fractions were translated in Xenopus laevis oocytes as described by Asselbergs et al. (1978). In short, RNA was dissolved in 10 to 20 µl distilled water and 40 nl of this solution was injected per oocyte. For each mRNA fraction 20 oocytes were injected and labeled for 20 h at 19 °C in 100 µl of modified Barth's medium containing 1 mCi/ml L-(^{35}S)methionine. Lysates were prepared by homogenizing the oocytes in 1 ml of immunoprecipitation buffer (see below). The lysates were cleared by centrifugation for 15 min at 220,000 x g and carefully aspirating the lipid containing upper layer.

Immunoprecipitation and polyacrylamide gel electrophoresis. Immunoprecipitation of radioactive polypeptides from lysates of metabolically labeled mouse cells and Xenopus oocytes was performed as described by Van Zaane et al. (1976) in 10 mM sodium phosphate-pH 7.2, 0.9 % NaCl, 1 % Triton X-100, 0.5 % sodium deoxycholate and 0.1 % SDS. Immunoprecipitation on lysates of iodinated cells was carried out in the same buffer, except that the SDS concentration was raised to 0.25 %. For precipitation of rabbit antisera we used Protein A-Sepharose CL-4B (50 µl of a 10 % (v/v) suspension per µl of antiserum). For precipitation of mouse antiserum either Protein A-Sepharose CL-4B or a rabbit-anti-mouse(IgG) serum was used. Immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to Laemmli (1970) on 0.75 mm thin 7 to 18 % polyacrylamide gradient slab gels. ^{3}H, ^{14}C or ^{35}S labeled protein bands were visualized after treatment of the gels with dimethylsulphoxide/2,5-diphenyloxazole (DMSO/PPO) (Bonner and Laskey, 1974) by scintillation autoradiography with preflashed Kodak XR films (Laskey and Mills, 1975). Autoradiographic detection of iodinated protein bands was accelerated by using Du Pont Cronex Lighting-Plus X-ray intensifying screens (Swanstrom and Shank, 1978).

RESULTS

Membrane immunofluorescence.

The presence of the Moloney cell surface antigen on several Mo-MuLV -infected and uninfected cell lines was analyzed by membrane immunofluorescence. The MCSA-positive Mo-MuLV -infected JLS-V9 cell line obtained from Dr E.M. Fenyö was used as a reference. The other cell lines studied were two different Mo-MuLV -infected NIH-3T3 lines - NIH-3T3(NCL5611P3)/Mo-MuLV and NIH-3T3/Mo-MuLV(clone 1A) -, and the uninfected NIH-3T3 and JLS-V9 parental lines. All lines were negative with the non-immune control sera. As expected, the uninfected cell lines were also negative with anti-MCSA. The Mo-MuLV infected cell lines all showed a positive fluorescence with anti-MCSA although the expression of MCSA on the Mo-MuLV -infected NIH-3T3 cells was much lower than that on the JLS-V9/Mo-MuLV cells.

Molecular characterization of Moloney cell surface antigen.

To characterize the polypeptide species recognized by anti-MCSA on the surface of Mo-MuLV -infected cells, these cells were surface-iodinated. As a control, uninfected cells were also iodinated. Lysates were prepared and immunoprecipitation was carried out with anti-MCSA and anti-MuLV. Immunoprecipitates were analyzed by means of polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). Of the infected cell lines only the JLS-V9/Mo-MuLV cells expressed MCSA in sufficient amounts to be readily detected by immunoprecipitation. This is in agreement with our results obtained by membrane immunofluorescence (see above). In Fig. 1 it is shown that both anti-MCSA (lane d) and anti-MuLV (lane c) precipitated polypeptides of 70,000 and 82,000 apparent molecular weight from the lysate of iodinated infected cells, though in different ratios. In the anti-MuLV precipitate the 70,000 molecular weight protein clearly predominates, whereas in the anti-MCSA precipitate the 70,000 and 82,000 molecular weight proteins are present in about equal amounts. A 70,000 molecular weight

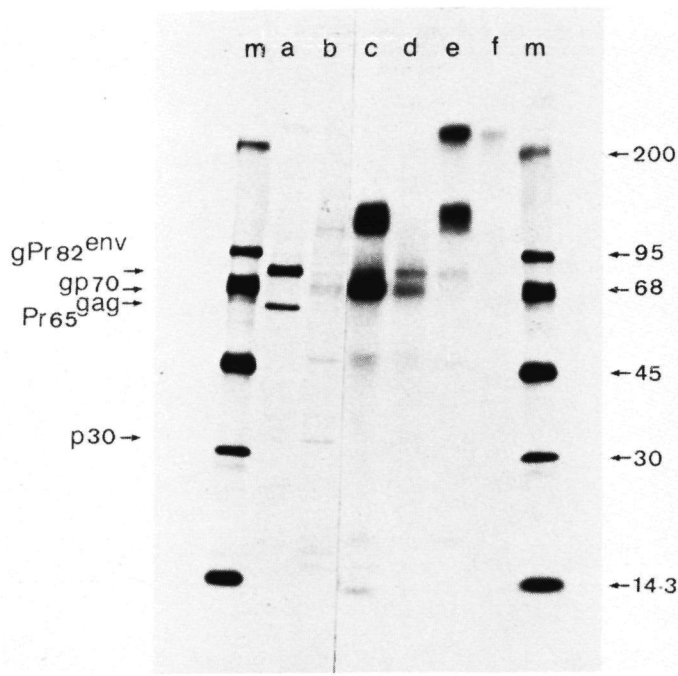


Figure 1. Molecular characterization of MCSA.
Mo-MuLV -infected and uninfected JLS-V9 cells were surface-labeled with ^{125}I. Lysates were prepared and immunoprecipitations were carried out with anti-MCSA and anti-MuLV. As a reference, to identify viral proteins, anti-MuLV precipitates from Mo-MuLV -infected NIH-3T3(NCL5611P3) cells, pulse-labeled for 1 h with (U-^{14}C)protein hydrolysate and chased for 20 h, were used. Precipitates were analyzed by SDS-PAGE followed by autoradiography. (a) ^{14}C pulse; (b) ^{14}C chase; (c,d) iodinated infected cells; (e,f) iodinated uninfected cells. Antisera: (a,b,c,e) anti-R-MuLV; (d,f) anti-MCSA. Lanes m contain ^{14}C-labeled molecular weight marker proteins. Molecular weights are indicated in thousands.

protein could not be precipitated from the lysate of uninfected cells (lanes e, f), but a low amount of an 82,000 molecular weight protein was present in the anti-MuLV precipitate of this lysate (lane e). This protein is completely absent from the anti-MCSA precipitate of uninfected cells (lane f). The 70,000 molecular weight protein comigrated on the gel with the viral envelope glycoprotein gp70, precipitated from the lysates of Mo-MuLV -infected cells labeled with ^{14}C-amino acids (lane b). Likewise, the 82,000 molecular weight protein comigrated with the env precursor polyprotein $gPr82^{env}$ (lane a; cf. next section). The 70,000 molecular weight protein precipitated by anti-MuLV from the lysate of iodinated infected cells is probably identical to gp70 since gp70 is expressed on all MuLV-infected cells (Famulari and Jelalian, 1979; Witte and Weissman, 1976). It is known that the anti-MCSA serum contains some anti-gp70 activity (Fenyö et al., 1977) and, therefore, the precipitation of the 70,000 molecular weight protein by anti-MCSA, which is relatively weak (compare lanes c and d), is presumably due to this contaminating activity in the antiserum. We tentatively concluded from these results that MCSA is present as a single protein with an apparent molecular weight of 82,000.

Biosynthesis and turnover of MCSA in Mo-MuLV -infected cells. To study the biosynthesis of MCSA, Mo-MuLV -infected cells were labeled with L-(^{35}S)methionine or with a ^{14}C-labeled amino acid mixture in pulse-chase experiments. Immunoprecipitates from cellular lysates obtained with anti-MCSA as well as with antiserum directed against viral polypeptides were analyzed by SDS-PAGE. As shown in Fig. 2 (lanes b), after a 15 min pulse, a protein with an apparent molecular weight of 82,000, $p82^{MCSA}$, could be precipitated with anti-MCSA from the lysates of all three Mo-MuLV -infected lines tested. This MCSA-positive protein comigrates on the gel with the MCSA molecule present on the cell surface (Fig. 1) and with viral precursor-polypeptides (Fig. 2, lanes a). By analysis with monospecific antisera these were shown to be the

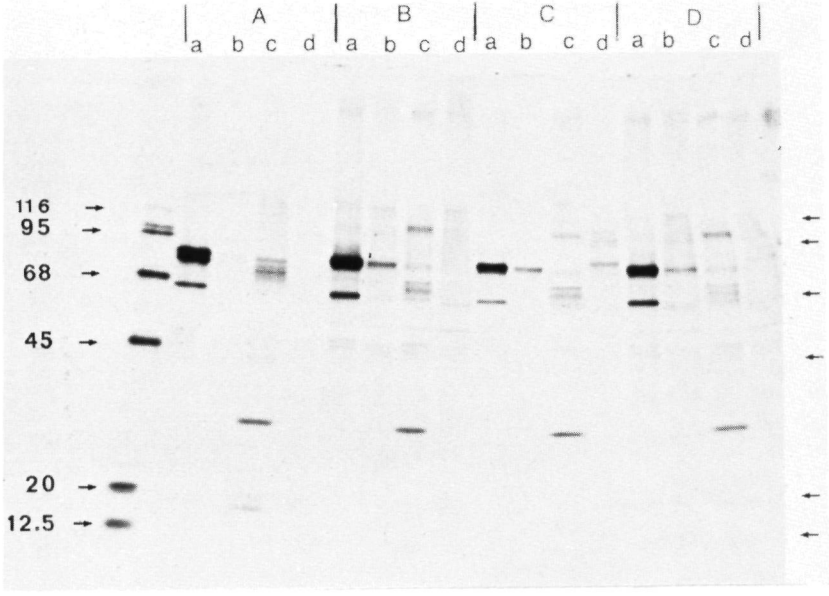


Figure 2. Biosynthesis and turnover of MCSA.
Mo-MuLV -infected cells were pulse-labeled for 15 min with L-(^{35}S)methionine or (U-^{14}C)protein hydrolysate and chased for 8 h. Lysates were prepared and immunoprecipitation was carried out with anti-MCSA or with anti-R-MuLV.
Precipitates were analyzed by SDS-PAGE followed by scintillation autoradiography. (A) ^{35}S-labeled NIH-3T3/Mo-MuLV(clone 1A) cells; (B) ^{35}S-labeled NIH-3T3(NCL5611P3)/Mo-MuLV cells; (C) ^{14}C-labeled NIH-3T3(NCL5611P3)/Mo-MuLV cells; (D) ^{35}S-labeled JLS-V9/Mo-MuLV cells. (a) Pulse, anti-R-MuLV; (b) pulse, anti-MCSA; (c) chase, anti-R-MuLV; (d) chase, anti-MCSA. ^{14}C-labeled proteins were used as molecular weight markers.

precursor polyprotein of the envelope proteins, $gPr82^{env}$, and the gag polyprotein $gP80^{gag}$ (results not shown; cf. Ledbetter et al., 1978; Van Zaane et al., 1976, Wood and Arlinghaus, 1979). Like $gPr82^{env}$, $p82^{MCSA}$ from NIH-3T3/Mo-MuLV(clone 1A) cells but not from NIH-3T3(NCL5611P3)/Mo-MuLV or JLS-V9/Mo-MuLV cells is sometimes seen as a double band (Fig. 2A, lanes a, b). A double band for $gPr82^{env}$ is found more often and was attributed to a variation in glycosylation (Van Zaane et al., 1976; Witte and Baltimore, 1979), although recent results indicate, that the heterogeneity of $gPr82^{env}$ (and gp70) can also be caused by the presence of multiple, closely related, genomes in the virus (Murray and Kabat, 1979).
After a chase of 8 h, $p82^{MCSA}$ had completely disappeared; no obvious processing product(s) could be found (Fig. 2, lanes d). Only faint bands are visible on the gel, the most prominent one being a band at 87,000 molecular weight. The viral precursor proteins $gPr82^{env}$ and $gP80^{gag}$ are chased to the envelope proteins gp70, p15E and p12E and to the glycosylation variants of $gP80^{gag}$, $gP95^{gag}$ and $gP85^{gag}$, respectively (Ledbetter et al., 1978; Van Zaane et al., 1976; Wood and Arlinghaus, 1979) (Fig. 2, lanes c). Presumably the anti-(Mo-MuLV)gp70 activity in the anti-MCSA serum is too low to be detectable in this case.
It should be noted here, that we were hardly able to detect the glycosylated gag polyproteins after amino acid labeling (Fig. 2, lanes c). This is in accordance with earlier work (Van Zaane et al., 1976). The band of 105,000 apparent molecular weight seen in anti-MuLV precipitates of chase lysates (Fig. 2, lanes c) is not gag-related for it was not precipitated by anti-p30 (not shown). Both anti-MuLV and anti-MCSA only very weakly precipitated some polypeptides from the lysates of uninfected cells. Moreover, none of these polypeptides comigrated with the viral (poly-)proteins or the MCSA-related protein that could be precipitated from infected cells, except for a 87,000 molecular weight protein that was precipitated by anti-MCSA from both infected and uninfected cells after an 8 h chase (results not shown).

According to the literature (Edwards and Fan, 1979; Ledbetter et al., 1978; Van Zaane et al., 1976), both $gPr82^{env}$ and $gP80^{gag}$ are glycosylated. It was already known that MCSA is also glycosylated (Siegert et al., 1977). The gag polyprotein can be labeled with mannose but not with glucosamine, whereas $gPr82^{env}$ (and gp70) is readily labeled with the latter carbohydrate (Edwards and Fan, 1979; Ledbetter et al., 1978; Van Zaane et al., 1976). We found that $p82^{MCSA}$ could be labeled with glucosamine (results not shown). An MCSA-positive protein was also detected in Rauscher murine leukemia virus (R-MuLV) -infected cells. The glycosylated gag polyprotein of R-MuLV clearly moves faster than the env precursor on the gel (Van Zaane et al., 1976). The MCSA protein found in JLS-V9/R-MuLV cells comigrated with the env precursor and not with the gag polyprotein on the gel (results not shown). From these introductory results we concluded that the main MCSA-positive polypeptide found in pulses of infected cells might be env-related. The processing of $p82^{MCSA}$ differs from the processing of Mo-MuLV polyproteins, indicating that MCSA is distinct from Mo-MuLV proteins.

Characterization of the MCSA-mRNA.

The existence of intracellular virus-specific mRNAs of different size classes can be demonstrated by translation of size-fractionated RNA from virus-infected cells in Xenopus laevis oocytes followed by immunoprecipitation and SDS-PAGE (Van Zaane et al., 1977). The same method enabled us to detect intracellular mRNAs that code for MCSA-positive products. $Poly(A)^+$ RNA from NIH-3T3(NCL5611P3) cells infected with Mo-MuLV was fractionated by centrifugation on isokinetic sucrose gradients. Samples from the fractions were injected into Xenopus oocytes. As a control, H_2O was injected. After incubation for 20 h in the presence of L-(^{35}S)methionine oocyte extracts were prepared and subjected to precipitation with either anti-MCSA or anti-MuLV followed by SDS-PAGE (Fig. 3 A, B). S-values of the injected mRNAs are indicated in the figure. Only a very low percentage (less than 0.1 %) of the proteins made in the

Figure 3. (A + B) (page 151).
Messenger RNA coding for MCSA.
Messenger RNA from Mo-MuLV -infected NIH-3T3(NCL5611P3) cells was isolated (100 µg) and size-fractionated on an isokinetic sucrose gradient. Samples of each fraction were injected into 20 oocytes of Xenopus laevis.
After labeling the oocytes with L-(^{35}S)methionine, immunoprecipitation was carried out on the lysates with anti-MCSA (3A) or with anti-R-MuLV (3B).
Precipitates were analyzed by SDS-PAGE followed by scintillation autoradiography.
Positions of 18S and 28S ribosomal RNA on a parallel gradient are indicated by big arrows. Small arrows indicate calculated S-values of RNA. Lanes "p+c" show the immunoprecipitable polypeptides found in the combined pulse and chase lysates of NIH-3T3/Mo-MuLV(clone 1A) cells. As a control, precipitates from lysates of oocytes injected with water are shown in lanes "H_2O". Lanes m contain ^{14}C-labeled marker proteins.

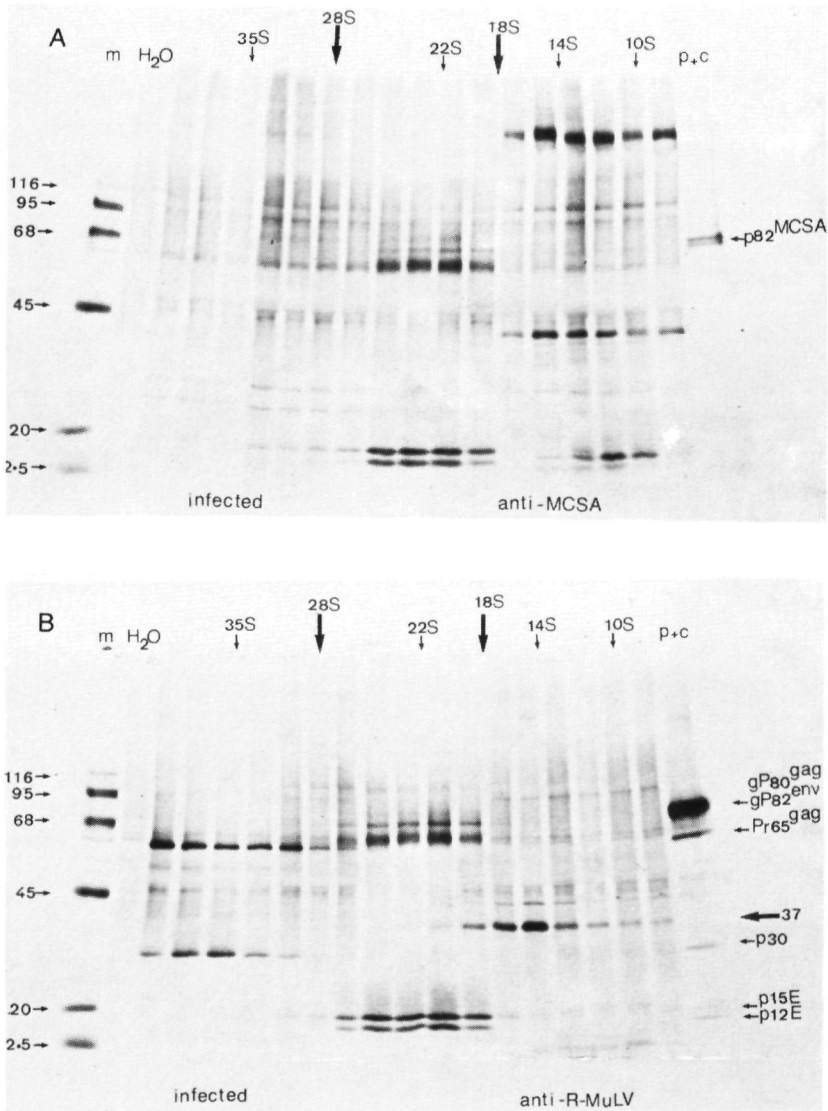


Figure 3. (A + B). Messenger RNA coding for MCSA.
(Legend on page 150).

injected oocytes are virus-specific. Therefore, a rather high background of aspecific protein bands is seen on the gels. The blank control serves to discern these aspecific bands from the immunoprecipitable proteins specifically made in response to the injected RNA. Fig. 3A shows the immunoprecipitates with anti-MCSA; the precipitates with anti-MuLV from the same lysates are shown in Fig. 3B. The results presented in Fig. 3B are similar to those reported and discussed by us before (Van Zaane et al., 1977). Apparently, 35S mRNA did not code for any protein precipitable with anti-MCSA. With anti-MuLV the gag (poly-)proteins $Pr65^{gag}$ and p30 are found in this RNA size region. Note, that p15, p12 and p10 can not be seen, because p15 and p10 lack methionine and p12 is not recognized by the antiserum used (cf. Van Zaane et al., 1976). Moreover, the gag polyproteins $gP80^{gag}$, $gP95^{gag}$ and $gP85^{gag}$ are not found in oocytes after labeling periods longer than 10 h (Salden et al., 1976; Van Zaane et al., 1977; F.A.M. Asselbergs, Ph.D. thesis, University of Nijmegen, Nijmegen, The Netherlands, 1979). Apparently, these proteins have a rapid turnover in oocytes and their messenger RNA is quickly degraded.

The $p82^{MCSA}$ molecule was synthesized on 22S mRNA. This molecule is processed in oocytes to proteins that comigrate exactly with gp70, p15E and p12E, precipitated with anti-MuLV from the same oocytes (compare Figs 3A and 3B). The perfect comigration of these four MCSA-positive proteins with the viral env proteins can be seen more accurately in Fig. 6.

The polypeptides that were precipitated in the 9-18S region by either anti-MCSA or anti-MuLV are not specific translation products of infected cell mRNAs. The protein with apparent molecular weight of 200,000 has a much higher molecular weight than corresponds to the size of the mRNAs in this region ($0.2 - 0.7 \times 10^6$) (Dudov et al., 1976). Other proteins (apparent molecular weights 50,000, 43,000, 37,000, 17,000, 13,000) could also be found by immunoprecipitation of the translation products of 9-18S uninfected cell mRNAs. Messenger RNAs of 35S and 22S size from uninfected cells

did not direct the synthesis of precipitable products (results not shown).

In the previous section some indications for the env-like nature of $p82^{MCSA}$ were described, although, unlike $gPr82^{env}$, $p82^{MCSA}$ was not chased into products comparable to gp70, p15E and p12E in the infected cells. In oocytes, however, precisely such a processing seems to occur. Moreover, the size of the mRNA coding for these MCSA-positive env-like molecules corresponds exactly with the size of the mRNA coding for the Mo-MuLV env proteins (compare the lanes between the 28S and 18S markers). This strongly emphasizes the env-like character of $p82^{MCSA}$.

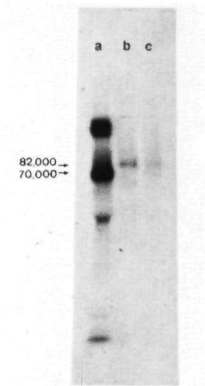
Competition experiments.

It was clearly shown by Fenyö and her colleagues that MCSA is unrelated to the Mo-MuLV structural proteins (Fenyö and Klein, 1976; Fenyö et al., 1977).

We were able to confirm this in competition experiments, carrying out immunoprecipitations in the presence of an excess of unlabeled viral proteins. In the first experiment we studied the effect of the addition of Moloney virus, supplemented with an extra amount of gp70, on the precipitation by anti-MCSA and anti-MuLV of iodinated surface proteins of Mo-MuLV -infected cells.

It follows from Fig. 4 that precipitation of gp70 by both anti-MuLV (lane c) and anti-MCSA (lane b) was inhibited completely, whereas the precipitation of the 82,000 molecular weight proteins remained unimpaired. Apparently, the surface-labeled gp70 is related to Mo-MuLV. The surface-labeled $p82^{MCSA}$, on the other hand, is absent in Mo-MuLV particles and serologically different. The lack of competition for the weak band of the 82,000 molecular weight protein in the anti-MuLV precipitate indicates that this protein does not represent Moloney $gPr82^{env}$. It can not be $p82^{MCSA}$, precipitated by cross-reacting antibody in the anti-MuLV serum, either since anti-MuLV also precipitates an 82,000 molecular weight protein present on the surface of uninfected cells (Fig. 1, lane e).

Figure 4. Competition of MCSA with MuLV proteins.
Immunoprecipitations on surface-iodinatec JLS-V9/Mo-MuLV cells were carried out in the presence of 100 /ug of Mo-MuLV and 1C /ug of (R-MuLV)gp70.
Precipitates were analyzed by SDS-PAGE followed by autoradiography. (a) Anti-R-MuLV, no addition; (b) anti-MCSA, plus virus and gp70; (c) anti-R-MuLV, plus virus and gp70.



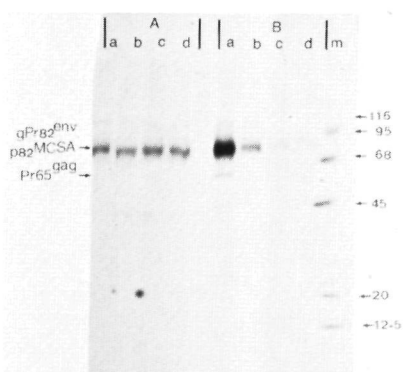


Figure 5. Competition of $p82^{MCSA}$ with MuLV proteins.
Lysates of NIH-3T3/Mo-MuLV (clone 1A) cells, labeled for 15 min with L-(^{35}S) methionine, were immunoprecipitated with anti-MCSA (A) or with anti-R-MuLV (B).
For competition, virus or viral proteins were added. (a) No addition; (b) 100 /ug of Mo-MuLV; (c) 10 /ug of (R-MuLV)gp70; (d) 100 /ug of Mo-MuLV and 10 /ug of (R-MuLV)gp70.
Precipitates were analyzed by SDS-PAGE followed by scintillation autoradiography. Lane m contains ^{14}C-labeled marker proteins.

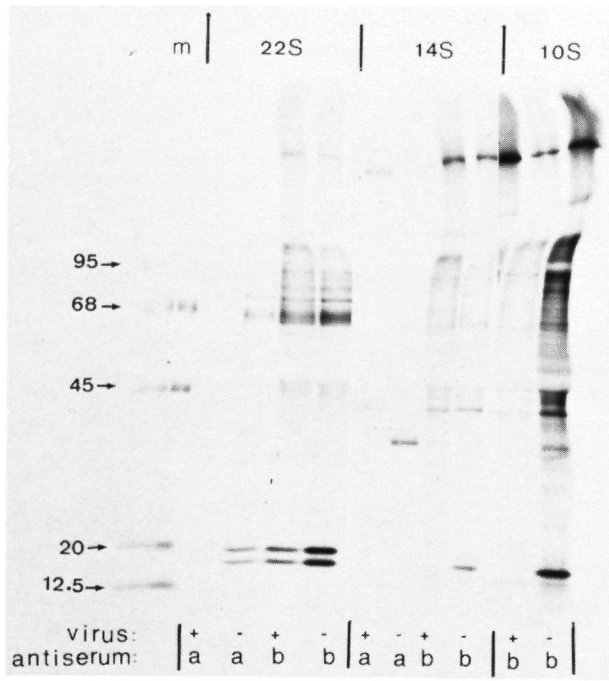


Figure 6. Competition of MCSA-positive oocyte translation products with Mo-MuLV proteins.
Lysates of oocytes injected with RNA of 22S, 14S and 10S size from NIH-3T3(NCL5611P3)/Mo-MuLV cells (cf. Fig. 3) were immunoprecipitated with anti-R-MuLV (a) or with anti-MCSA (b), in the presence (+) or absence (-) of 100 µg of Mo-MuLV.
Precipitates were analyzed by SDS-PAGE followed by scintillation autoradiography. Lane m contains ^{14}C-labeled marker proteins.

Similar results were obtained in competition experiments using the lysate of pulse-labeled infected cells (Fig. 5). The Moloney virus preparation apparently contained a low amount of viral envelope proteins, probably due to the isolation procedure (see Methods section), since virus alone only partially inhibited the precipitation of $gPr82^{env}$ by anti-MuLV under these circumstances (lane B.b). In contrast, labeled $Pr65^{gag}$ was lost completely from the precipitate (lane B.b). Added gp70 competed better with the env precursor polyprotein (lanes B.c, d). Precipitation of the MCSA-positive protein was not affected by the addition of unlabeled Mo-MuLV and/or gp70 (lanes A.a - d).

When unlabeled Mo-MuLV was added to the immunoprecipitations of oocyte translation products it completely blocked the precipitation of env-proteins by anti-MuLV, but had no influence on the precipitation of the MCSA-related env-products (Fig. 6). These results indicate that in Xenopus oocytes $p82^{MCSA}$ is processed to mature "env-like" proteins and that "gp70-like" and "p15E,p12E-like" molecules were not precipitated by a contaminating anti-Mo-MuLV activity in the anti-MCSA serum. Apparently, $p82^{MCSA}$ is expressed on the cell surface in such a way that it is able to elicit antibodies to its gp70 moiety as well as to the p15E portion of the molecule. The proteins that could be precipitated by anti-MuLV or anti-MCSA from the translation products of 9-14S mRNA from either infected or uninfected cells had cross-reacting counterparts in the virus (Fig. 6). Supposedly, these are cellular proteins, associated with the viral proteins on or close to the cell surface and thus encapsidated in the virion during the budding process. These proteins are not present in large quantities in the cells (Fig. 2), nor are they exposed on the cell surface well enough to be labeled with iodine (Fig. 1).

In summary, the competition experiments show that the main activity in the anti-MCSA serum is directed at env-like proteins of a type-C virus serologically different from Mo-MuLV.

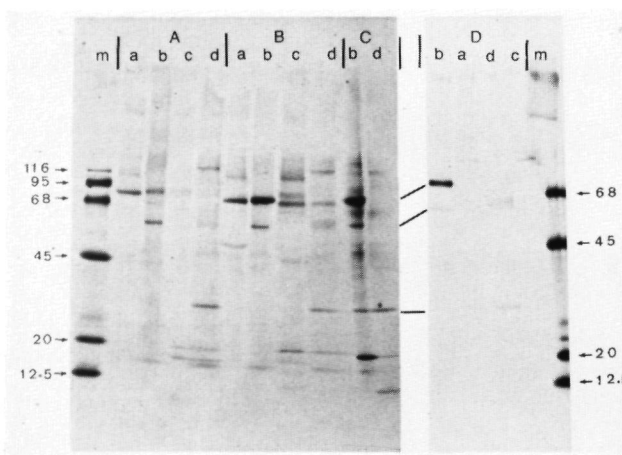


Figure 7. Relationship of MCSA to the envelope proteins of prototype viruses.
AKR virus-infected NIH-3T3 cells (A), (AKR)MCF-247 virus-infected SC-1 cells (B) and NZB xenotropic virus-infected CCL64 cells (D) were pulse-labeled for 15 min with L-(^{35}S)methionine and chased for 8 h.
Immunoprecipitates were applied on SDS-PAGE and radioactivity was traced by scintillation autoradiography. (a) Pulse, anti-MCSA; (b) pulse, anti-R-MuLV; (c) chase, anti-MCSA; (d) chase, anti-R-MuLV. As a reference, immunoprecipitates with anti-R-MuLV from NIH-3T3(NCL5611P3)/Mo-MuLV cells, labeled for 1 h with L-(^{35}S) methionine (C.b) or chased for 20 h (C.d) were used. Lanes m contain ^{14}C-labeled marker proteins.

Reactivity of anti-MCSA with proteins of prototype viruses.

To further investigate the specificity of the anti-MCSA serum, we carried out immunoprecipitations on lysates of pulse-chase labeled cells that were infected with three different prototype viruses. These viruses were the N-ecotropic AKR virus (Fig. 7, A), the N-amphotropic (AKR)MCF-247 virus (B) and the xenotropic NZB virus (D). These were all three cloned virus isolates. The (AKR)MCF-247 virus is an env gene recombinant between ecotropic AKR virus and a xenotropic virus (Elder et al., 1977; Rommelaere et al., 1978). The gag sequences of the recombinant are AKR-related, whereas part of the env sequences are of xenotropic origin. Lysates of NIH-3T3 (NCL5611P3) cells infected with Mo-MuLV were used as a reference (C). The anti-MuLV serum recognized gag and env proteins of all three prototype viruses (lanes b, d). Although the corresponding envelope (precursor) proteins of the four viruses presented in Fig. 7 have about the same molecular weight, slight differences in their electrophoretic mobilities can be seen. This has been reported before (Famulari and Jelalian, 1979; Wood and Arlinghaus, 1979). Clearly (lanes a, c), anti-MCSA reacted with the envelope (precursor) proteins $gPr82^{env}$, gp70, p15E and p12E of both AKR virus and (AKR)MCF-247 virus, but did not recognize NZB xenotropic virus proteins.

DISCUSSION

Most of the work on MCSA has been performed with the Mo-MuLV -induced YAC lymphoma cell line (cf. Introduction). We used Mo-MuLV -infected fibroblast cell lines. Yet, we could not have missed antigens that are present on the YAC cells and not on the infected fibroblast lines, because in two of the three infected cell lines (JLS-V9/Mo-MuLV and NIH-3T3(NCL5611P3)/Mo-MuLV) the Moloney virus was originally derived from the culture fluid of YAC cells (Fenyö and Klein, 1974); this virus transfers MCSA activity (Grundner et

al., 1974) and it was shown that JLS-V9 cells infected with this virus absorb all YAC-cell -directed cytotoxic activity in the anti-MCSA serum (Grundner et al., 1974). This does not necessarily mean that MCSA is present on the same molecule(s) in YAC lymphoma cells as it is in Mo-MuLV -infected fibroblast cells. On the latter cells MCSA is expressed as a single glycoprotein of 82,000 apparent molecular weight (our results). Molecular weights for the YAC cell-derived MCSA have been reported before (Siegert et al., 1977; Troy et al., 1977). In those experiments MCSA was detected by inhibition of the cytotoxic activity of an anti-MCSA serum. By lectin-affinity chromatography followed by gel filtration MCSA appeared to be present on a glycoprotein with a molecular weight of 110,000 (Siegert et al., 1977). On SDS gels MCSA activity was predominantly found at a position of 90,000 apparent molecular weight, with minor peaks at 52,000 and 180,000-190,000 (Troy et al., 1977). The minor peaks (each representing less than 1 % of the main activity peak; Troy et al., 1977) were not detected by us.

The accuracy of molecular weight estimations by gel filtration is limited and molecular weights estimated by different techniques or by different investigators with the same technique may vary to some extent.

Particularly, variation in the reported molecular weights of the MuLV envelope precursor polyprotein should be noted in this context (Naso et al., 1976; Van Zaane et al., 1976).

Therefore, we conclude that our estimate of the molecular weight of MCSA on fibroblasts is not in disagreement with the estimates of others of the molecular weight of lymphocytic MCSA.

Since we detected MCSA-positive molecules in JLS-V9 cells and NIH-3T3 cells infected with the YAC-derived virus as well as in NIH-3T3 cells infected with Mo-MuLV from a different source, the presence of MCSA is apparently not restricted to a certain cell type nor is the MCSA-inducing capacity restricted to one particular virus isolate. (Note, that R-MuLV is also able to induce MCSA; Fenyö and Klein, 1976, and our results). No large differences were

observed in the MCSA content of the three cell lines studied (Fig. 2). On the other hand, the expression of MCSA on the cell surface was highly cell-dependent: both by membrane immunofluorescence and by surface iodination MCSA was hardly detectable on the Mo-MuLV -infected NIH-3T3 cells but could readily be detected on JLS-V9/ Mo-MuLV cells. This difference in the surface expression of MCSA might be related to the different origin of 3T3 and JLS-V9 cells: 3T3 cells are of whole embryo origin (Todaro and Green, 1963), whereas JLS-V9 cells were derived from BALB/c bone marrow (Wright et al., 1967). Both cell lines essentially have a fibroblastic appearance, however.

Our conclusion that MCSA is related to MuLV envelope proteins is based on a number of observations: the messenger for MCSA has the same size (22S) as the mRNA coding for MuLV envelope proteins (Fig. 3); the MCSA-positive molecule found in infected cells ($p82^{MCSA}$) comigrates on SDS gels with the envelope precursor polyprotein $gPr82^{env}$ (Figs 2, 7); $p82^{MCSA}$ is labeled by glucosamine as is $gPr82^{env}$; in cells where $gPr82^{env}$ is present as a double band on the gel, $p82^{MCSA}$ is also double on the gel (Fig. 2, lanes a, b); and, finally, in oocytes $p82^{MCSA}$ is processed to proteins comigrating on SDS gels with gp70, p15E and p12E (Fig. 3).

The oocyte system is an excellent system for translation of eukaryotic mRNAs since it is the only translation system known that is able to adequately perform all steps in the processing of primary translation products, like proteolysis and glycosylation. Remarkably, oocytes sometimes process proteins of which the processing is blocked *in vivo*. For instance, it is known, that *in vivo* processing of the MuLV *gag* precursor polyprotein occurs concomitantly with virus budding (Yeger et al., 1978). Clearly, *Xenopus* oocytes do not produce virus when injected with 35S mRNA from MuLV-infected cells; yet they do process the *gag* precursor polyprotein, $Pr65^{gag}$, correctly (Fig. 3B). Therefore, $p82^{MCSA}$, although not processed in the infected cell, could very well be processed in the oocyte to MCSA-related mature *env* proteins (Fig. 3A).

Apparently, processing of the MCSA-positive envelope precursor polyprotein is inhibited in the infected cells, since we were not able to detect significant amounts of processing products in these cells (Fig. 2, lanes d). This indicates that MCSA is not identical to Mo-MuLV proteins. This conclusion was substantiated by the results of the competition experiments. A 50- to 100-fold excess of unlabeled Moloney virus or (R-MuLV)gp70 blocked the immunoprecipitation of radioactive Mo-MuLV proteins, but did not influence the precipitation of MCSA (Figs 4, 5, 6). These results fully agree with the results obtained by others, who also showed that MCSA was distinct from Mo-MuLV structural proteins (cf. Introduction). Neither was MCSA related to the so-called FMR cell surface antigen that is found on cells infected with Friend, Moloney or Rauscher-MuLV, since this antigen was shown to be an env determinant that is also present in virions of the FMR group (Nowinski et al., 1978). Since MCSA is related to MuLV env proteins but serologically different from the envelope proteins of Mo-MuLV, the question arises: are there viruses that carry MCSA determinants?

We performed immunoprecipitations with anti-MCSA on cells infected with three prototype viruses to investigate whether anti-MCSA would recognize any one of these viruses (Fig. 7). The results show that anti-MCSA recognized AKR ecotropic virus as well as (AKR)MCF-type virus envelope proteins. Both the envelope precursor polyproteins and the mature envelope proteins were recognized.

Anti-MuLV sera raised in mice are predominantly directed at type-specific antigenic determinants (Brown et al., 1979). However, although (AKR)MCF virus is an env-gene recombinant between AKR ecotropic virus and a xenotropic virus, the fact that anti-MCSA did not recognize NZB xenotropic viral proteins does not necessarily mean that MCSA consists of a set of ecotropic viral env antigenic determinants because it is not yet known which xenotropic virus is the parental virus of (AKR)MCF virus, and anti-MCSA could very well recognize antigenic determinants of xenotropic viruses other than the NZB virus.

The anti-R-MuLV serum has a broad specificity (Fig. 7). It precipitated gag and env (poly-)proteins of all three prototype viruses. This antiserum apparently recognizes other, probably group-specific, env determinants than anti-MCSA does, since its activity could be absorbed completely by Mo-MuLV, in contrast to the activity of the anti-MCSA serum that was not absorbed at all by Mo-MuLV (Figs 4, 5, 6).

Because anti-MCSA recognizes AKR virus envelope proteins one would expect Gross-MuLV -induced lymphomas to cross-react immunologically with Mo-MuLV -induced lymphomas. Preimmunization with irradiated G-MuLV -induced tumor cells did not protect against small isografts of Mo-MuLV -induced tumors, however (Klein and Klein, 1964).

But, although not quite understood, G-MuLV -induced lymphomas predominantly evoke cytotoxic antibodies against the glycosylated gag polyproteins present on the cell surface (Snyder et al., 1977). Apparently they do not induce a significant env-directed immune response.

In an attempt to further characterize the putative MCSA-related virus we tried to use monoclonal anti-MuLV antibodies that we had received from Dr R.C. Nowinski. We obtained prototype samples of six antibody classes (based on their different reactivity with a series of murine and non-murine viruses) (Lostrom et al., 1979). These antibodies were either directed against gp70 or p15E. Only two antibody classes did not recognize Mo-MuLV. These two classes did not detect the presence of another C-type virus when used in immunoprecipitations on lysates of the Mo-MuLV -infected cell lines (results not shown). Therefore, it is unlikely, that the putative virus responsible for the expression of MCSA is an N-ecotropic virus (like the AKR virus), since this group of viruses is recognized by these antibody classes (Lostrom et al., 1979). Clearly, more work has to be done to identify the nature of the putative MCSA-producing virus. Competition experiments using different (prototype) viruses to absorb anti-MCSA antibodies, would be one possibility to do this.

How does the expression of another C-type virus gene in Mo-MuLV -infected cells arise? One explanation could be, that the expression of an endogenous virus was induced by the infection with Mo-MuLV. This seems rather unlikely, for that would mean that everytime cells are infected with Mo-MuLV, the same virus would be induced. Moreover, NIH-3T3 cells are known for the fact that they do not contain inducible viruses (Aaronson and Stephenson, 1976).

A more plausible explanation is that the MCSA producing virus is present as a pseudotype in the Mo-MuLV isolates. Although we do not have a direct proof for this assumption, it is consistent with our results as well as with all other data reported in the literature.

1. It is known that non-clonal Mo-MuLV stocks contain a number of different viruses in addition to the ecotropic exogenous Moloney virus (Troxler et al., 1977b). Particularly, MCF-type viruses, with env-regions related to the env-region of (AKR)MCF-virus and to that of the spleen focus-forming component (SFFV) of the exogenous Friend and Rauscher virus strains are found (Shih et al., 1978; Troxler et al., 1977a; Troxler et al., 1977b).

2. Since $p82^{MCSA}$ is expressed on the cell surface (Fig. 1) and not processed to mature "env-like" proteins (Fig. 2) and since no MCSA-related env proteins are present in the Moloney virus isolate (Figs 4, 5, 6), the putative pseudotype virus coding for MCSA would be a replication-defective virus depending on the helper functions provided by an other virus (Mo-MuLV). The R-MuLV isolate could also contain the same (or a closely related) virus.

3. The envelope precursor polyprotein of most murine leukemia viruses is not found on the surface of infected cells (Famulari and Jelalian, 1979; Van Zaane et al., 1976; Witte and Weissman, 1976), but Famulari et al. (1979) showed that, typically, MCF-type viruses do express their env-precursor on the cell surface.

4. Karande et al. (1979) found that MCSA was associated with MuLV gag proteins on the cell surface. This could indicate the presence of blocked budding virus particles containing the MCSA-positive envelope precursor polyprotein and gag precursor polyproteins of

either Mo-MuLV origin or derived from the putative MCSA producing virus. The gag protein p15 rather stably associates with viral envelope proteins (Van de Ven et al., 1978). Similarly, co-chromatography of MCSA and p15 was found (Karande et al., 1979; Siegert et al., 1977).

5. As found by Grundner et al. (1974), immunoselection of cells that did no longer express MCSA led to the selection of cells producing a variant of Mo-MuLV that was unable to induce MCSA on indicator cells. If our assumption is correct, this would mean that Grundner et al. (1974) have obtained a Mo-MuLV isolate that is not contaminated with the pseudotype virus responsible for MCSA expression.

The significance of MCSA is its relation to the tumor-associated transplantation antigen (TATA) of Mo-MuLV -induced lymphomas (Klein et al., 1966). Since it is known that viral envelope proteins (including SFFV/MCF-type virus envelope proteins) present on the cell surface evoke both humoral and cellular immune responses (Boiocchi and Nowinski, 1979; Gillis et al., 1979; Kende et al., 1979), MCSA might well be identical to TATA.

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7. CAN 35S VIRUS-SPECIFIC RNAs BE SPLICED IN NUCLEI OF *XENOPUS LAEVIS* OOCYTES ?

CAN 35S VIRUS-SPECIFIC RNAs BE SPLICED IN NUCLEI OF XENOPUS LAEVIS OOCYTES ?

ABSTRACT

Purified 35S poly(A)$^+$ genomic RNA of Moloney murine leukemia virus was injected into the nuclei of Xenopus oocytes, in order to establish whether this RNA could be spliced into subgenomic RNA synthesizing env-related proteins. By coinjection of complete virions and purified viral proteins the potential role of the virus proteins in the splicing process was investigated. Furthermore, poly(A)$^-$ 35S viral RNA, decapped 35S poly(A)$^+$ viral RNA, and 35S cytoplasmic and nuclear RNA from infected cells were also injected. In all cases only gag proteins were synthesized and no env proteins could be detected. We also failed to generate functionally active env-mRNA by endonucleolytic fragmentation of genomic RNA with RNAse III.

Our results differ in part from those of others obtained in a different system. Possible reasons for this apparent discrepancy are discussed.

INTRODUCTION

The first reports on mRNAs consisting of contiguous segments that are coded for by widely separated portions of the genomic DNA were dealing with SV-40 and adenovirus-2 (3,6,22,29). This phenomenon has since been reported for a large number of mRNAs of animal viruses and eukaryotic cellular mRNAs (1,39). Apparently, eukaryotic genes usually consist of sequences that are represented in the cytoplasmic mRNA (expressed regions or "exons"; 20), intertwined with regions that are lost from the mature mRNA (intragenic regions or "introns"; 20).

A stretch of DNA, sometimes consisting of more than one gene (e.g. SV-40, adenovirus, retrovirus), is transcribed into one large precursor-mRNA complementary to the complete DNA sequence (12,20, 52). This primary transcription product can be found in the nucleus in the so-called heteronuclear RNA (hnRNA) fraction (12,24,33,56). In several steps this RNA is processed to the mature mRNA that is transported from the nucleus to the cytoplasm (reviewed in references 1,12,39,52). During or immediately after synthesis one molecule of guanosine ("cap") is invertedly coupled to the 5' end of the primary transcript through a 5'-5' triphosphate bond (19, 46,56). Subsequently methyl-groups are attached to the newly acquired guanosine and its adjacent nucleotide and to internal adenosines at specific sites in the RNA molecule (48,54). Concomitantly, the 3' end of the RNA is often polyadenylated (33). The modified RNA is now trimmed in one or more steps (a process generally referred to as "splicing") to one or more mature mRNAs by excision of the sequences complementary to the introns and ligation of the remaining sequences (complementary to the exons) (20,29). Splicing offers a mechanism by which one piece of DNA could contain several overlapping genes: differential splicing could lead to more than one mRNA, coding for different proteins.

The splicing phenomenon has also been observed for RNA tumor viruses.

The env-mRNA of the avian and mammalian leukemia viruses as well as the src-mRNA of the avian sarcoma virus have their 5' sequence (of at least 150 nucleotides in MuLV; 21,43) in common with the 35S genomic RNA, but lack the intervening sequences between the common leader and the gene for which the mRNAs code (for references: see chapter 2.4.5.). So in the env-mRNA the gag and pol genes and in the avian src-mRNA the gag, pol and env sequences are deleted. In the nucleus no MuLV-specific RNAs larger than genome size were detected (18,23). The main representative of nuclear virus-specific RNA had a size of 35S, but small amounts of 22S (env-) mRNA were also found. Therefore it is guessed that the primary transcript of retrovirus proviral DNA, apart from modifications like capping, methylation and polyadenylation described above, is identical to the genomic RNA and that the subgenomic mRNAs are generated in the nucleus. Small differences in the sequences of the presumed primary transcription product and the genomic RNA present in the virions can not be excluded, however. In particular, the possible presence in the primary transcript of the long terminal repeats that are contained by the proviral DNA (cf. chapter 2.3.) should be considered in this context. Similarly, subtle differences between cytoplasmic virus-specific 35S mRNA and the virion RNA might exist (cf. chapter 2.4.3.). Such differences, undetected sofar, could have functional significance.

Oocytes were shown to synthesize virus-specific tumor antigens and capsid proteins correctly when their nuclei were injected with SV-40 or polyoma DNA (30,44). Intranuclear injection of cloned Drosophila histone DNA also resulted in the synthesis of the correct protein product (13). This implies the correct splicing of at least part of the corresponding mRNAs, even though the majority of the transcripts might not be synthesized or processed accurately (26). Correctly spliced RNA-products were formed from cloned yeast $tRNA^{trp}$ genes in an in vitro system prepared from oocyte nuclei (1). It should be stressed here, however, that no successful

experiments have been reported on *in vitro* splicing of precursor-mRNA by injection of this RNA into nuclei of oocytes and subsequent analysis of either the mRNA itself or the protein products. Assuming that the 35S RNA present in MuLV virions is identical to the primary viral transcription product found in infected cells, we investigated the ability of the oocyte nucleus to splice genomic MuLV RNA into subgenomic *env*-mRNA. Detection was indirect, namely by immunoprecipitation with anti-gp70 serum of virus-specific translation products. From an other point of view this experiment could also be regarded as a test on genomic RNA being the right substrate for splicing. Similar experiments were performed with 35S cytoplasmic RNA and with nuclear RNA from infected cells.

In parallel we used *E. coli* RNAse III in order to investigate whether functional *env*-mRNA might be enzymatically produced from genomic MuLV RNA. The idea to use RNAse III for this purpose was based on earlier observations of Leis et al. (29) in the ASV system and of Shanmugam (47) in the MuLV system which indicated that RNAse III is able to degrade genomic RNA into discrete subgenomic RNAs (28) and that an enzyme with similar properties is present in mouse cells (47).

MATERIALS AND METHODS

Materials, cells and virus. RNAse III from *E. coli* was obtained from Enzo Biochem. Inc., New York. Tobacco acid pyrophosphatase (TAP) came from Bethesda Research Labs. Inc., Bethesda, Md. DNAse I was from Worthington Biochem. Corp., Freehold, N.J. A DNAse I preparation that was purified from RNAse contaminations by UTP-agarose chromatography (49) was a gift of Mr C.A.G. van Eekelen. R-MuLV proteins p10 and p12 were a gift of Dr J.R. Stephenson. Mo-MuLV, isolated from concentrated medium of roller-bottle cultures of NIH-3T3/Mo-MuLV(clone 1A) cells by the method described by

Duesberg and Robinson (14), was a gift of Dr A.J.M. Berns. Before injection into oocytes the virus preparation (5 mg/ml) was quickly frozen (CO_2/aceton) and thawed (37 °C) five times. All other materials, cells and virus are described in the corresponding sections of chapters 4 and 6.

Viral RNA. Purified viral 35S RNA from clone 1A Moloney virus was a gift of Mr C.J.M. Saris.

In short, RNA was liberated from the virus by treatment with proteinase K in the presence of SDS (0.5 %) and EDTA (1 mM) and size-fractionated on an isokinetic sucrose gradient in 10 mM Tris-pH 6.9, 100 mM NaCl, 1 mM EDTA and 0.2 % SDS. After heat-denaturation the 50-70S RNA fraction was poly(A)-selected by oligo(dT)-cellulose chromatography (5) and 35S RNA was isolated by centrifugation on an isokinetic sucrose gradient in low salt buffer as described in chapter 4.

Cellular mRNA. The isolation of 35S mRNA from NIH-3T3(NCL5611P3)/Mo-MuLV cells is described in detail in chapter 4.

Nuclear RNA. Several methods were used to isolate high molecular weight nuclear RNA from NIH-3T3(NCL5611P3)/Mo-MuLV cells (see Results and discussion section).

Cells were labeled for 16 h with 5 μCi of (2-^{14}C)thymidine to monitor contaminating DNA and, after preincubation for 20 min with 0.04 μg/ml actinomycine D (to stop the ribosomal RNA synthesis; 37), labeled for 10 min with 100 μCi of (5,6-^{3}H)uridine (to follow the fate of the hnRNA during the isolation procedure). The method that gave the best results (see Results and discussion section) is described here.

Solutions and glassware were sterilized (cf. chapter 4). Indicated pH values are at 25 °C. All procedures were carried out at 0 - 2 °C, unless stated otherwise. Cells were removed from the culture flasks by scraping in calcium- and magnesium-free Tyrode's solution containing 4 mM EDTA. The cells were washed twice with isotonic buffer (10 mM Tris-pH 7.2, 146 mM NaCl) and resuspended in 10 volumes RSB (10 mM Tris-pH 7.0, 10 mM NaCl, 1.5 mM $MgCl_2$).

Tween-40 and sodium deoxycholate were added in a concentrated mixture to final concentrations of 1 % and 0.5 %, respectively, and lysates were prepared by forcing the cells through a 21 gauge needle (36). The nuclei were pelleted by centrifugation for 10 min at 1200 x g and washed once with RSB. Then the nuclei were resuspended to the original lysate volume in 10 mM Tris-pH 8.2, 2 mM EDTA and 0.5 % SDS, a same volume of saturated phenol was added and RNA was extracted at 55 °C for 20 min (45,53). Subsequently, the water-phase was extracted twice with phenol/chloroform/isoamylalcohol (100 : 100 : 1) and once with chloroform/isoamylalcohol (25 : 1) at room temperature. RNA was precipitated from the water-phase by the addition of one volume of 6 M NaAc-pH 5.2, dissolved in H_2O and alcohol-precipitated twice.

The nuclear RNA was poly(A)-selected and size-fractionated as described for cellular RNA in chapter 4.

Decapping of viral RNA. The methylated "cap" of purified 35S poly(A)$^+$ viral RNA was removed by treatment with tobacco acid pyrophosphatase (TAP) (47). Conditions were identical to those indicated by the supplier of the enzyme. A reaction volume of 10 µl contained: 50 mM NaAc-pH 5.2, 10 mM 2-mercaptoethanol, 1 mM EDTA, 10 µg of 28S rat lens rRNA (free of mRNA-activity; a gift of Dr F.A.M. Asselbergs), 2 µg of viral RNA and 2 units TAP. The mixture was incubated for 1 h at 37 °C. The reaction was stopped by the addition of 200 µl of 0.1 M NaAc-pH 5.2, 1 mM EDTA and 0.5 % SDS. The mixture was extracted once with phenol/chloroform at room temperature (35) and the extracted RNA was alcohol-precipitated.

RNAse III-treatment of viral RNA. A reaction volume of 50 µl contained (41): 20 mM Tris-pH 7.4 (at 37 °C), 0.1 mM EDTA, 10 mM $MgAc_2$, 0.1 mM dithioerythritol, 5 % sucrose, 0, 50 or 100 mM KCl, 0.2 µg of purified 35S poly(A)$^+$ viral RNA and 2 units of RNAse III. The mixtures were incubated for 5 - 60 min at 37 °C. The reaction was stopped by the addition of 150 µl of 0.1 M NaAc-pH 5.2, 5 mM EDTA and 0.5 % SDS, containing 10 µg of 28S rat lens rRNA. The mixture was extracted once with phenol/chloroform (35)

and the extracted RNA was alcohol-precipitated.

Injection of oocytes. Intracytoplasmic injections of oocytes were performed as described earlier (cf. chapter 4). Intranuclear injections were carried out by the method of Kressman et al. (25). Oocytes were centrifuged on a nylon sieve (mesh size 0.8 mm) for 10 min at 200 - 400 x g with their animal poles (dark hemispheres) facing upwards. The site of the nucleus could now be localized by the displacement of pigment granules, giving rise to a lighter spot on the oocyte surface, caused by the flotation of the nucleus towards the surface wall.

RNA and proteins were injected in a volume of 15 nl per nucleus.

Immunoprecipitation. Oocyte lysates were prepared and immunoprecipitations were carried out as described in chapter 4.

RESULTS AND DISCUSSION

Injection of genomic viral RNA into oocyte nuclei.

Purified 35S poly(A)$^+$ viral RNA was injected into the cytoplasm and into the nucleus of oocytes. After injection the oocytes were preincubated in modified Barth's medium (4) for 16 h and thereafter labeled for 40 h with L-(^{35}S)methionine. Immunoprecipitation with anti-gp70 was performed on the complete lysate of 20 injected oocytes. On the supernatant of the first immunoprecipitation a second immunoprecipitation with anti-MuLV was carried out. Results are shown in Fig. 1. As found earlier by our group and many others, 35S viral RNA only directed the synthesis of gag proteins (for references see chapter 2.4.6.). In cell-free systems only the precursor polyproteins are made, but oocytes are able to process these polyproteins adequately (lanes a', b'; cf. ref. 4 and chapters 3,4,5,6). In cell-free systems the gag-pol precursor polyprotein is made in addition, but in oocytes pol-related (poly)proteins can not be seen under these circumstances (4; chapter 4). Apparently oocyte nuclei were not able to process the

35S viral RNA into functional 22S env-mRNA (lane a). Variation of the labeling conditions (starting the labeling immediately after injection and continuing for 16, 40 or 64 h) also did not result in the production of env proteins (not shown).
After injection of 35S viral RNA into the nucleus gag proteins were synthesized, although in lower amounts than after injection into the cytoplasm (compare lanes a' and b'). Therefore, part of the intranuclearly injected viral RNA must have entered the cytoplasm. It is not clear whether this was due to active transport of the viral RNA from the nucleus to the cytoplasm, to leakage of the injected nuclei or to simply a certain percentage of misses, where we had failed to hit the nucleus.

Coinjection of viral proteins and genomic viral RNA into oocyte nuclei.

Some viral proteins are able to interact with genomic RNA (for references: see chapter 2.2.). The phosphoprotein pp12 specifically binds to the homologous genomic RNA, whereas the basic protein p10 binds to single-stranded nucleic acids in general. A functional significance for these properties is not yet known. We wanted to consider a possible function of these proteins in the splicing process. Therefore, we coinjected viral 35S RNA and the proteins p10 and p12 and also genomic RNA with disrupted Mo-MuLV into oocyte nuclei and, as a control, into the cytoplasm. Labeling and immunoprecipitation on samples of the lysates was performed as described in the previous section. Again, injection of poly(A)$^+$ 35S viral RNA, either in the nucleus or in the cytoplasm, only resulted in the synthesis of gag proteins (Figs 2A and 2B, lanes a, a'). Coinjection of pp12 did not change the results (lanes f, f'). Coinjection of p10, however, completely abolished translational activity (lanes e, e'). The effect of p10 could be prevented by the addition of pp12 (lanes g, g'). Finally, disrupted Mo-MuLV altered the translational pattern of 35S viral RNA (lanes d, d'): independent of the site of injection of the RNA a protein with apparent

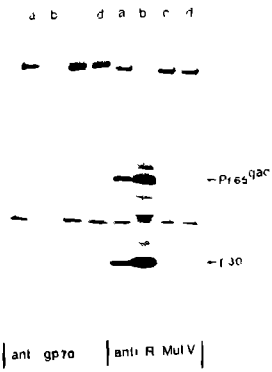

Figure 1. Injection of genomic viral RNA.
Purified 35S poly(A)$^+$ Mo-MuLV RNA (1 mg/ml) was injected into the nucleus and into the cytoplasm of Xenopus oocytes (15 nl per oocyte). Oocytes were preincubated in modified Barth's medium for 16 h and labeled for 40 h with L-(^{35}S)methionine. Translation products were first immunoprecipitated with anti-gp70. On the supernatant of this immunoprecipitation a second precipitation with anti-MuLV was carried out. Precipitates were analyzed by SDS-PAGE followed by scintillation autoradiography. Lanes a-d, anti-gp70; lanes a'-d', anti-MuLV. (a,a') RNA injected into the nucleus; (b,b') RNA injected into the cytoplasm; (c,c') H_2O injected into the nucleus; (d,d') H_2O injected into the cytoplasm.

Figure 2.(A + B) (page 181).
Injection of modified genomic RNA. Coinjection of genomic RNA and viral proteins.
Mo-MuLV RNA (0.5 mg/ml) was injected into the nucleus or in the cytoplasm of Xenopus oocytes (15 nl per oocyte). Oocytes were preincubated for 16 h and labeled for 40 h with L-(^{35}S)methionine. Translation products were analyzed by immunoprecipitation followed by SDS-PAGE and scintillation autoradiography. (A) anti-gp70; (B) anti-MuLV. Lanes a-g, intracytoplasmic injection; lanes a'-g', intranuclear injection. (a,a') 35S poly(A)$^+$ RNA; (b,b') 35S poly (A)$^-$ RNA; (c,c') 35S poly(A)$^+$ RNA treated with tobacco acid pyrophosphotase; (d,d') 35S poly(A)$^+$ RNA coinjected with disrupted Moloney virus (2.5 mg/ml); (e,e') coinjection with 0.5 mg/ml p10; (f,f') coinjection with 0.5 mg/ml p12, (g,g') coinjection with p10 and p12; (m) ^{14}C-labeled molecular weight marker proteins (cf. chapter 4, Fig. 1.).

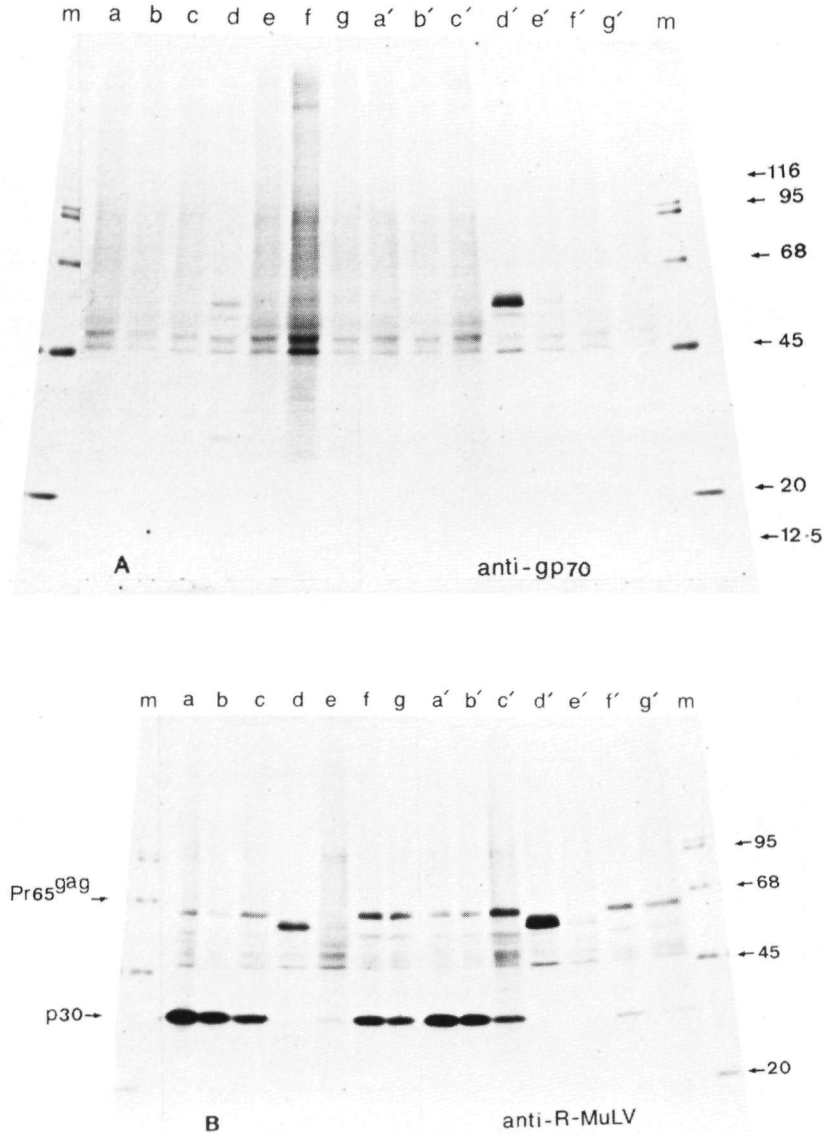

Figure 2. (A + B). Injection of modified genomic RNA. Coinjection of genomic RNA and viral proteins. (Legend on page 180).

molecular weight of 60,000, called tp60[1], was synthesized that could be precipitated by anti-MuLV. Moreover, only after intracytoplasmic injection, a second protein, with an apparent molecular weight of 28,000, tp28, was detected with anti-MuLV. At first sight (Fig. 2B, lanes d, d') it seems as if $Pr65^{gag}$ and p30 were slightly modified by some component of the virus. This can not be true since these proteins were precipitable with anti-gp70 (Fig. 2A, lanes d, d'). Because this was the only situation in which env-related proteins could be precipitated after injection of genomic RNA into oocytes we performed some additional experiments (Fig. 3). The Mo-MuLV preparation did not influence immunoprecipitation when added, even in a tenfold higher concentration than it was present in the coinjection experiment, to the lysate of oocytes injected with poly(A)$^+$ 35S viral RNA only (Fig. 3, lanes a, a') or when injected without viral RNA (in the latter case no virus-specific products could be immunoprecipitated; not shown). From Fig. 3 it can be seen further that tp60 and a protein of about 10,000 apparent molecular weight, tp10 (not visible in Fig. 3), could be precipitated from lysates of oocytes, coinjected with viral RNA and Mo-MuLV, by anti-p30 (lanes b, b'), anti-gp70 (lanes c, c') and anti-p15E,p12E (lanes d, d'). These proteins were also precipitable with anti-BSA (lanes e, e') and, therefore, this precipitation should be regarded as non-specific. Only tp28 (lane b) and a protein of 55,000 apparent molecular weight, tp55, (lanes b, b'; also weakly visible in Fig. 2A, lanes d, d') and a protein of 8,000 apparent molecular weight, tp8, (lanes b, b'; not seen in Fig. 2A, lanes d, d') seem to be precipitated specifically by anti-gp70. The molecular weights of these polypeptides do not equal those of known env-related (poly-)proteins. We have not studied this bizarre phenomenon any further.

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1) tp = translational product; see chapter 5.

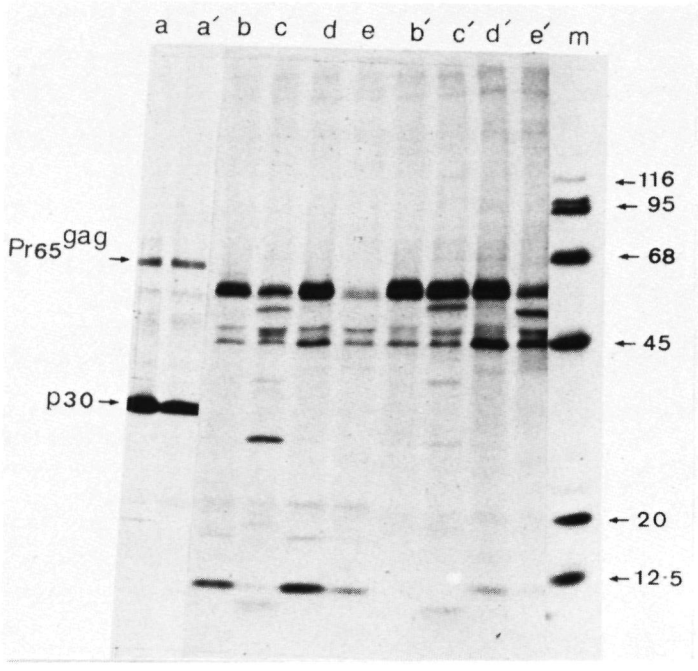

Figure 3. Influence of Mo-MuLV proteins on the translation of Mo-MuLV RNA in oocytes.
Purified 35S poly(A)+ Mo-MuLV RNA was injected alone (lanes a,a') or coinjected with disrupted Moloney virus (lanes b,b'-e,e') into the nucleus and into the cytoplasm of Xenopus oocytes. Oocytes were preincubated for 16 h and labeled for 40 h with L-(^{35}S) methionine. Translation products were analyzed by immunoprecipitation followed by SDS-PAGE and scintillation autoradiography. Lanes a-e, intracytoplasmic injection; lanes a'-e', intranuclear injection. Precipitation was with: (a,a') anti-MuLV in the presence of Moloney virus (7.5 µg/ml lysate); (b,b') anti-p30; (c,c') anti-gp70; (d,d') anti-p15E,p12E; (e,e') anti-BSA.

Injection of modified genomic viral RNA into oocyte nuclei.

The scheme of nuclear mRNA processing depicted in the Introduction section is a generalized one and individual variations may exist, dependent on the mRNA studied (12,52).

Therefore we also injected poly(A)$^-$ 35S viral RNA and decapped poly(A)$^+$ genomic RNA into oocyte nuclei. As can be seen from Fig. 2 (lanes b, b', c, c') these modifications did not influence translation qualitatively and only slightly reduced translation efficiency. This latter effect, if significant at all, could reflect the increased instability reported for mRNAs without poly(A) or cap (39).

RNAse III treatment of viral RNA.

The endoribonuclease RNAse III from E.coli has a functional role in the processing of ribosomal RNA precursors in bacteria (16), but the best characterized series of processing reactions of this enzyme involves the early mRNA precursor of bacteriophage T7 (15). RNAse III acts on native RNA by nicking unpaired nucleotide sequences in the vicinity of double-stranded RNA, e.g. a "hairpin loop" structure. Cleavage is not only dependent on structure but also depends on the sequence in the "hairpin loop" (42). In addition to these "primary cleavage sites" (17) at non-physiological low salt concentrations or at high enzyme : substrate ratios "secondary", but still highly specific sites are cleaved by RNAse III (17,34, 42,55). Data sofar obtained suggest that the secondary cleavage reaction of RNAse III provides the equivalent of an RNA "restriction enzyme", allowing reproducible production of specific RNA fragments.

An RNAse III-like activity has been detected in mammalian and avian cells (1,47) which might be involved in the processing of rRNA- and mRNA-transcription units (1,12).

Retroviral RNA has a high order of secondary structure (10,27,40). Therefore we treated genomic viral RNA with RNAse III in an attempt to generate subgenomic RNA molecules that would have the initiation

site for env protein synthesis exposed. Both salt conditions and incubation time were varied. In all cases translational activity was lost and no env proteins were synthesized when the RNAse III-treated RNA samples were injected into the cytoplasm of oocytes. Accordingly the size of the treated RNA had changed from 30-35S to the low molecular weight range (below 7S) as measured on polyacrylamide gels (courtesy of Mr C.J.M. Saris). Apparently, MuLV genomic RNA is not cleaved by RNAse III as specifically as was found with some E. coli RNAs (17,34,42,55). We did not further test the enzyme at limiting enzyme : substrate ratios which might result in the production of larger fragments (11).
Stacey et al. who used mild alkali-treatment (50) or limited RNAse T1 digestion (51) to generate large fragments from Rous-associated virus-2 genomic RNA were not able to measure any env-mRNA activity of these fragments in a very sensitive system (cf. chapter 2.4.5,8). Genomic RNA might therefore be an inappropriate substrate for the endonucleolytic generation of env-mRNA (e.g. because it might lack the env initiation site; this does not exclude other 35S virus-specific RNA specimens from being suitable substrates). Alternatively, the env initiation site might only be produced by a process of splicing and not by mere degradation of larger RNAs. In contrast, Purchio et al. (38) were able to generate active src-mRNA from 38S ASV genomic RNA by RNAse T1 treatment.

Injection of cytoplasmic 35S virus-specific RNA into oocyte nuclei.
Not only virion RNA, also cytoplasmic virus-specific 35S RNA failed to be spliced into active env-mRNA in oocyte nuclei.
Intranuclear injection of poly(A)$^+$ 35S cytoplasmic RNA did not result in the synthesis of env proteins. As with 35S viral RNA only gag proteins were synthesized (results not shown).

Injection of nuclear RNA into oocyte nuclei.
Isolation of high molecular weight nuclear RNA is hard to achieve. Several criteria should be fulfilled: 1. The nuclear RNA should be

free of cytoplasmic RNA contaminations. To satisfy this condition cells should be lysed in such a way as to leave the nuclei intact (9). Nuclei can be further purified by mixed-detergent treatment and/or centrifugation through 0.88 M sucrose (7,9). 2. The nuclear RNA should be free of contaminating DNA. This aim can be achieved by DNAse treatment (7,36) or by phenol/chloroform extraction at 55 ^{o}C ("hot phenol"; 7,31,45,53). Additionally, in high salt (3 M NaAc or 2 M LiCl) single-stranded RNA is precipitated but DNA remains dissolved (7,35). Absolute care should be taken that the DNAse preparation to be used is free of RNAse activity. Commercial DNAse I preparations, though called "RNAse-free", are still contaminated with RNAse (8). They can be further purified by affinity chromatography on UMP-agarose (8) or UTP-agarose (49). "Hot phenol" extraction in the presence of SDS inactivates ribonuclease instantly (7). 3. The nuclear RNA should remain intact. It is known that different cell types have different levels of intracellular ribonucleases (7; S. Penman and J. Darnell, personal communication). The major source of RNAse are the lysozomes (7) that fall apart by detergent lysis as well as by mechanical disruption of the cells. The most powerful RNAse-inhibitors known, SDS and heparin, can not be included in lysis buffers since they cause desintegration of the nuclei. Moreover they also inhibit DNAse action.

To optimize the isolation of high molecular weight nuclear RNA from Mo-MuLV -infected NIH-3T3 cells we used several methods. The method that gave the best results is described in the Methods section. This method resulted in a nuclear RNA preparation that was still contaminated with less than 2 % of the DNA originally present in the cells (determined by ^{14}C-thymidine labeling). The size of hnRNA, labeled by a short ^{3}H-uridine pulse, was determined by isokinetic sucrose gradient centrifugation and ranged between 7 and 50S, with a broad maximum between 20 and 40S. This size distribution did not significantly change after poly(A)-

selection of the RNA. The size of isolated unlabeled steady-state nuclear RNA (primarily consisting of (pre-)rRNA) ranged between 4 and 50S with a broad maximum between 20 and 35S. No discrete peaks were found at 45S, 28-32S and 18S, as can be seen with Hela cell nuclear RNA (36). Our results are in line with those of Kamen et al. (2) who isolated nuclear RNA from polyoma virus-infected NIH-3T3 cells.

As total and poly(A)-selected nuclear RNA was translated in oocytes by intracytoplasmic or intranuclear injection no virus-specific polypeptides could be precipitated. These results are only seemingly in contrast with those of Kamen et al. (2) who detected polyoma virus-specific (late) protein synthesis in a wheat germ cell-free system with nuclear RNA that was not purified further, since it should be realized that late polyoma virus-specific RNA contributes up to 10 % of the hnRNA (2), compared to only 0.5 % for MuLV-specific RNA (18), and that the four times smaller polyoma virus late transcripts are less prone to degradation than the 35S MuLV primary transcripts. Our experiments do not exclude that fractionated nuclear RNA would contain enough translatable material to detect the synthesis of virus-specific proteins.

CONCLUSIONS

Our negative results with genomic viral RNA as a template for splicing into env-mRNA in oocyte nuclei are in agreement with the results of Asselbergs et al. (4). These investigators used complete (70S and heat-denatured) viral RNA from R-MuLV isolated from the plasma of viremic mice. They did not detect env-mRNA activity in these preparations, no matter whether these were injected into the cytoplasm or the nucleus of the oocyte. This is in contrast with the finding of Stacey who detected env-mRNA activity in the virion RNA of RAV-2 virus (51). This activity was demonstrated by intra-cytoplasmic injection of the RAV-2 virion RNA into cells infected with an env-deletion mutant of RSV. Complementation of the deficient

function was detected by the release of focus-forming virus (cf. chapter 2.4.5,8). The discrepancy between the results of Asselbergs et al. and those of Stacey may be explained by the higher sensitivity of the assay used by the latter author, in whose system the response is magnified by virus multiplication. The same explanation might be used for our apparent failure to generate env-mRNA from purified 35S viral RNA by injection into the nucleus of oocytes, whereas Stacey and Hanafusa were able to accomplish this splicing step by intranuclear injection of RAV-2 35S genomic RNA into (env-) BH-RSV -infected cells (50). Since in this latter system gag and pol proteins are present, one might argue that one or more of these proteins are necessary for the splicing of genomic RNA. This could be excluded, however, (cf. chapter 2.4.5.) by the results of intranuclear injection of 35S RAV-2 genomic RNA into chick embryo fibroblasts which expressed no viral functions. In this case also infectious (RAV-2) viruses were synthesized (50). Moreover, this result completely refutes the objection raised by Asselbergs et al. (4) that the env-mRNA in the intranuclearly injected BH-RSV -infected cells might have been generated from integrated proviral DNA, originated from the reverse transcription of the genomic RAV-2 RNA by reverse transcriptase molecules present in these cells. The apparent inability of Stacey et al. (50,51) to generate env-mRNA from genomic RAV-2 RNA chemically or enzymatically in vitro also made a non-specific formation (i.e. without splicing) of env-mRNA in vivo very unlikely.

Taken together all our results we conclude that the Xenopus oocyte nucleus is probably not a suitable system to study splicing of retroviral RNA, either because the sensitivity of the system is too low or because the oocyte nuclei are not able to handle the viral RNA properly to make it accessible for the splicing machinery (e.g. binding of essential factors to the RNA or attachment of the RNA to the nuclear skeleton might be required). Moreover, if an "RNAse III-like" enzyme is involved in the

processing of retroviral RNA *in vivo* at all, this enzyme should exhibit certainly more specificity than the *E. coli* enzyme did with this RNA.

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SUMMARY

This thesis deals with the expression of murine leukemia virus proteins. In chapter 1 our results are placed in the perspective of the rapidly evolving RNA tumor virus research of which chapter 2 gives a general survey.
Chapters 3 to 7 describe our experimental data.
In chapter 3 it is shown that the murine leukemia virus core proteins are synthesized on a genome-size (35S) virus-specific mRNA, whereas the viral envelope proteins are made on a subgenomic (22S) mRNA, comprising about the 3' half of the genome. These results were obtained by translation of size-fractionated mRNAs from infected cells in oocytes of the African clawed frog Xenopus laevis, immunoprecipitation of translation products from lysates of injected oocytes with polyvalent and monospecific antisera directed against viral proteins, and analysis of precipitated products by SDS-polyacrylamide gel electrophoresis. By the same method it was established in chapter 4 that the viral enzyme reverse transcriptase is also made on genome-size mRNA. Despite of much effort, we were not able to confirm these results independently by enzymatic detection of reverse transcriptase in mRNA-injected oocytes. In chapter 5 it is shown that translation of 14S mRNA in Xenopus oocytes resulted in the synthesis of presumably cellular proteins that were precipitable with antisera directed against complete murine leukemia virus and that are encapsidated by the virus particles.
Since it was known from the literature that the subgenomic mRNA directing the synthesis of murine leukemia virus envelope proteins is generated from genome-length RNA in the nucleus of infected cells by a splicing process, we attempted to mimic this process by injection of purified 35S poly(A)$^+$ RNA, 35S decapped poly(A)$^+$ RNA and 35S poly(A)$^-$ RNA from Moloney murine leukemia virus particles into the nuclei of Xenopus oocytes. This is shown in chapter 7.

We also studied the potential influence of viral proteins on the splicing process. In no case, however, we were able to detect the synthesis of envelope proteins. Yet we observed an alteration of the translation pattern, induced by some viral protein(s). In order to investigate whether we might have offered the wrong substrate for splicing, we directed our efforts to cytoplasmic 35S virus-specific mRNA and to nuclear RNA. These RNA species were equally inproductive in envelope protein synthesis after intranuclear injection into oocytes.

Our negative results with genomic RNA contrast with the positive results of others obtained in a different system. Possible explanations are discussed.

Positive results were obtained by the use of the oocyte translation system in the identification of the Moloney cell surface antigen (MCSA). This antigen is induced on mouse cells by Moloney murine leukemia virus and is related to the Moloney virus-induced tumor-associated transplantation antigen. We showed in chapter 6 that MCSA is an envelope gene product of a defective murine RNA tumor virus not related to the Moloney strain of murine leukemia virus.

SAMENVATTING

Het muize-leukemievirus behoort tot de RNA tumor virussen. Vertegenwoordigers van deze groep virussen komen voor bij zowel hogere als lagere gewervelde dieren. Sinds in 1970 een uniek enzym ontdekt werd in deze virussen, het zgn. "reverse transcriptase", dat in staat is het virale RNA genoom over te schrijven in DNA, is over de gehele wereld een intensief onderzoek gestart naar de bouw, de replicatie en de gen-expressie van de RNA tumor virussen en natuurlijk naar de wijze waarop zij tumoren kunnen veroorzaken. Hoofdstuk 1 van dit proefschrift plaatst onze experimentele gegevens, die in de hoofdstukken 3 tot en met 7 weergegeven worden, in het perspectief van het zich snel ontwikkelende RNA tumor virus-onderzoek, waarvan hoofdstuk 2 een, noodzakelijkerwijs beperkt, verslag geeft. De volgende hoofdstukken betreffen alle de expressie van muize-leukemievirus eiwitten, zoals ook aangegeven door de titel van het proefschrift. In de hoofdstukken 3 tot en met 5 wordt aangetoond dat de verschillende virale eiwitten gemaakt worden op boodschapper-RNA's van verschillende grootte. De eiwitten van het kerndeeltje van het virus en het "reverse transcriptase" worden gesynthetiseerd op genoom-lengte RNA terwijl de virale envelop eiwitten gemaakt worden op een boodschapper-RNA dat maar half zo groot is. Op een nog kleiner boodschapper-RNA werden eiwitten gesynthetiseerd van cellulaire oorsprong die op de één of andere manier in het virusdeeltje terechtkomen. Deze resultaten werden verkregen door het "vertalen" van boodschapper-RNA's die geïsoleerd waren uit geïnfecteerde cellen, in eicellen van de Afrikaanse klauwkikker Xenopus laevis. Deze eicellen zijn zo groot, dat zij met eenvoudige middelen te injecteren zijn, en hebben het voordeel ten opzichte van zgn. "celvrije" vertaalsystemen dat alle cellulaire processen ongestoord kunnen blijven verlopen. De virus-specifieke vertaal-producten werden geïsoleerd door middel van specifieke antisera en geanalyseerd met behulp

van de gel-elektroforese techniek.
Omdat uit de literatuur bekend was dat het RNA van subgenoom-grootte waarop de envelop eiwitten gesynthetiseerd worden ontstaat uit genoom-lengte RNA in de kern van geïnfecteerde cellen via een zgn. "splicing"-proces, hebben wij geprobeerd (hoofdstuk 7) of het mogelijk was dit proces na te bootsen door injectie van genoom-lengte virus-specifiek RNA in de kernen van Xenopus eicellen.
Onze pogingen bleven zonder succes. Mogelijke verklaringen hiervoor worden besproken.
Wel succesvol was het gebruik van het Xenopus eicel vertaal-systeem bij de karakterisatie van het "Moloney Cell Surface Antigen" (MCSA). Dit is een cel-oppervlakte antigeen dat door Moloney muize-leukemievirus op muize-cellen wordt geïnduceerd en dat nauw gerelateerd is aan het door het Moloney virus geïnduceerde tumor-specifieke transplantatie-antigeen.
Lange tijd is de aard van dit antigeen onbekend gebleven, maar in hoofdstuk 6 wordt aangetoond dat het hier een envelop eiwit betreft van een vooralsnog onbekend defectief virus dat niet gerelateerd is aan het Moloney virus.

CURRICULUM VITAE

Wim Hesselink werd op 18 februari 1951 te Winterswijk geboren. In 1969 behaalde hij het diploma gymnasium B aan het Thorbecke Lyceum te Arnhem. Daarna studeerde hij scheikunde aan de Rijksuniversiteit te Utrecht. In 1972 werd het kandidaatsexamen S2 afgelegd en op 22 september 1975 slaagde hij voor het doctoraal examen (cum laude). Keuzerichting was analytische chemie (prof.dr. A. Dijkstra) en bijvakken waren biofysische chemie (dr. R. van Wijk) en klinische immunologie (prof.dr. R.E. Ballieux). Van 1 september 1975 tot en met 31 december 1979 was hij in dienst van de Katholieke Universiteit te Nijmegen ten laste van de Nederlandse Organisatie voor de Kankerbestrijding/Koningin Wilhelmina Fonds (projectnummer NUKC-1975-7) en was hij werkzaam op het Laboratorium voor Biochemie van de Faculteit der Wiskunde en Natuurwetenschappen (hoofd: prof.dr. H. Bloemendal).
In de werkgroep van prof.dr. H.P.J. Bloemers verrichtte hij onderzoek aan de gen-expressie van muize-leukemievirussen, waarvan onder andere dit proefschrift de neerslag vormt.
Vanaf 1 februari 1980 is hij werkzaam op het Centraal Laboratorium van de Bloedtransfusiedienst van het Nederlandse Rode Kruis te Amsterdam, om als "fellow" van het Koningin Wilhelmina Fonds zich verder te bekwamen in de immunologische aspecten van het kankeronderzoek, met name dat van haematologische maligniteiten.

STELLINGEN

1.

De hoeveelheid virus-specifiek RNA dat door middel van nucleïnezuur-hybridisatie gedetecteerd kan worden in RNA tumor virus geïnfecteerde cellen, is ongeveer omgekeerd evenredig met de mate waarin dit RNA gepolyadenyleerd is.

Dit proefschrift, hoofdstuk 2.4.3.

2.

Bij het aangeven van het mogelijke belang van virale eiwitsynthese voorafgaand aan het integratieproces van RNA tumor virussen, vergeten Shurtz *et al.*, dat geen *de novo* synthese nodig is voor de introductie in de cel van eiwitten die bij de infectie al binnengekomen zijn, en dat defectieve sarcoma virussen die geen enkel leukemie virus specifiek eiwit kunnen produceren, zeer wel in staat zijn als provirus in het gastheer-genoom te integreren.

Shurtz, R., S. Dolev, M. Aboud en S. Salzberg. 1979. J. Virol. *31*: 668-676.

3.

Het idee dat de verhoogde H-2D expressie door thymocyten van muizen geïnfecteerd met het "radiation leukemia virus" (RadLV) veroorzaakt zou worden door integratie van het virus naast het H-2D locus is een oversymplificatie.

Meruelo, D. 1980. J. Immunogen. *7* : 81-90.

4.

De fysische associatie van RNA tumor virus eiwitten met histocompatibiliteitsantigenen op het oppervlak van geïnfecteerde cellen is een omstreden zaak. Het zicht hierop wordt niet verhelderd, wanneer Azocar en Essex ter adstructie van de door hen gevonden evidentie vóór associatie een artikel van Aoki *et al.* aanhalen, dat echter uitsluitend bewijs tégen het bestaan van deze associatie bevat.

Azocar, J. en M. Essex. 1979. Cancer Res. *39* : 3388-3391.
Aoki, T., E.A. Boyse, L.J. Old, E. DeHarven, U. Hämmerling en H.A. Wood. 1970. Proc. Natl. Acad. Sci. USA *65* : 569-576.

5.

Door het elektroforetisch patroon van verschillende eiwitten in natriumdodecylsulfaat/polyacrylamide gels ook in verschillende figuren weer te geven is het klaarblijkelijk mogelijk eiwitten die comigreren toch een met 10.000 verschillend molecuulgewicht toe te kennen.

Buetti, E. en H. Diggelman. 1980. J. Virol. *33* : 936-944.

6.

De H-2 restrictie van de immuunreactie bij muizen wordt niet bepaald door het H-2 fenotype van de thymus.

7.

Het door Williamson voorgestelde model voor de "major histocompatibility complex" (MHC) -geassocieerde herkenning van antigenen door T-lymfocyten biedt geen redelijke verklaring voor het functioneren van de in het MHC gelocaliseerde immuunresponse genen en is daardoor, althans in deze vorm, niet acceptabel.

Williamson, A.R. 1980. Nature (London) *283* : 527-532.

8.

De uitspraak van Present *et al.* dat 6-mercaptopurine bij langdurige toediening aan patiënten waarschijnlijk niet carcinogeen is, is slechts gebaseerd op de resultaten van een tweejarige observatie-periode en daarom voorbarig.

Present, D.H., B.I. Korelitz, N. Wisch, J.L. Glass, D.B. Sachar en B.S. Pasternack. 1980. New Engl. J. Med. *302* : 981-987.

9.

De veelbelovende studies naar het effect van immunisatie tegen *Streptococcus mutans* antigenen in de bestrijding van tandbederf (cariës) moeten krachtig voortgezet worden, al verdient het kweken van betere eetgewoontes toch de voorkeur.

Lehner, T., M.W. Russel en J. Caldwell. 1980. The Lancet *1* : 995-996.

10.

Het vinden van verschillen tussen de concentratieverhoudingen van androsteron en etiocholanolon in de urine van heterosexuelen en van homosexuelen heeft gelukkig geen invloed gehad op de voortschrijdende acceptatie van homosexuelen in onze samenleving.

Margolese, M.S. en O. Janiger. 1973. Br. Med. J. *3* : 207-210.

11

Kwangjoe, Kwangdjoe, Kwangju, Kwansju Kwanchu provincie-hoofdplaats in Zuid-Korea
Park Chung-Hee, Park Sjung-Hie voormalig Zuidkoreaans president
(voorbeelden uit dagbladen en NOS t v -journaal in de periode van 19 t/m 24 mei 1980)

Zolang voor de translitteratie van eigennamen vanuit een vreemd schrift naar het Latijnse schrift nog geen vaste regels gelden zoals bij het Chinees, dient hiervoor in het Nederlandse taalgebied toch in ieder geval consequent de Nederlandse spellingswijze gevolgd te worden

12

De regering dient thans actief de verspreiding van informatie over het vóór of tegen van kernenergie te bevorderen, om te voorkomen dat de door haar toegezegde, zo belangrijke maatschappelijke discussie over dit onderwerp, slechts "breed" in de zin van "oeverloos" zal worden

Nijmegen, 1 oktober 1980 Wim Hesselink